Development and evaluation of DNA vaccines in chickens against a wild bird H6N2 avian influenza virus from Western Australia

This thesis is presented for the degree of Doctor of Philosophy at Murdoch University

by

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Declaration

I declare that this thesis is my own account of my research and contains work which has not previously been submitted for a degree at any tertiary educational institution.

…………………….
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Abstract

Genetic immunization, also known as DNA or polynucleotide immunisation, is well documented to induce broad-based immunity in various animal models of infectious and non-infectious diseases. However, the low potency of DNA vaccines has to date precluded the development of commercial vaccines. The aim of this study was to systematically investigate a number of parameters to improve the potency of DNA vaccines for use in chickens, using a low pathogenic avian influenza (LPAI) virus as a proof-of-concept for their ability to produce a humoral immune response.

The index virus used in the study was avian influenza virus A/coot/WA/2727/79 (H6N2), isolated from an apparently healthy Eurasian coot in 1979. Prior to any DNA experiments the virus was rigorously characterized. The virus strain was shown to be an H6 subtype by haemaglutination inhibition (HI) testing and as an N2 subtype by gene sequence analysis. The isolate was shown to be able to grow on MDCK cells in the absence of exogenous trypsin. It was further biologically characterized as LPAI with an intravenous pathogenicity index (IVPI) of 0.15 and a motif of ^{321}PQAETRG^{328} at the cleavage site of the haemagglutinin (HA) protein. It was capable of infecting domestic chickens under experimental conditions with a low level of virus excretion via the cloaca and oropharynx following intravenous or oral and oculonasal inoculation.

The full-length HA and nucleoprotein (NP) genes of this H6N2 virus were subsequently cloned into the eukaryotic expression vector VR1012 to generate VR-HA and VR-NP constructs. Six-week-old Hy-Line chickens were intramuscularly injected with either the VR-HA or VR-NP vaccine at different dose rates, with or without lipofectin as adjuvant.

Minimal or no detectable antibody was produced, as measured by HI, ELISA and Western blotting-based assay, but high titres of H6-specific HI antibodies appeared 10 days after homologous virus challenge. In contrast to the empty vector controls, there was a significant difference in HI antibody titre between pre- and post-challenge in vaccinated birds, indicating some evidence for the priming effect of the DNA vaccines. Using the frequency of virus shedding as an indicator of protection, lower doses (50 or 100 μg per chicken) of either adjuvanted VR-HA or VR-NP vaccine significantly reduced virus shedding in oropharyngeal and cloacal swabs compared to higher doses (300 or 500 μg per chicken) or empty vector control chickens. Although two vaccinations with naked VR-HA alone were not sufficient to induce an effective immune response against a homologous virus challenge, further repeat vaccinations and incorporation of adjuvant did lead to the generation of low to moderate HI antibody titres in some chickens and resulted in no or reduced virus shedding after challenge.

Next, to examine the effect of expression vector, three different DNA vectors, pCI, pCI-neo and pVAX1 were used to clone the same HA gene and generate three DNA vaccine constructs. Once again, direct intramuscular injection of the three DNA constructs did not elicit measurable H6-specific HA antibody response in Hy-Line chickens but the 100 μg pCI-HA lipofectin adjuvanted vaccine group showed a significant increase in post-challenge HI titres from the naive control group, indicating that an anamnestic antibody response had been induced by the pCI-HA DNA vaccination. Compared with the controls, the three DNA constructs showed significantly reduced virus shedding in cloacal swabs post virus challenge, suggesting that the three DNA vaccines induced some level of immune response in vaccinated chickens. As with the VR-HA construct, the lower dose

groups for each vaccine (50 or 100 µg) were more effective at reducing virus shedding from the cloaca than the higher dose group (300 µg).

To further investigate why the DNA vaccines did not elicit a measurable antibody response, the HA gene incorporating a Kozak enhancer sequence was cloned into an alternative expression vector, pCAGGS, to produce the pCAG-HAk construct. Three-week-old SPF chickens were immunized with this construct either by the intramuscular route (IM) or electroporation (EP). H6 HI antibodies were present in some chickens by 3 weeks after the first IM vaccination and 75% of the chickens vaccinated with 10, 100 or 300 µg pCAG-HAk were antibody positive by 2 weeks after the second IM vaccination. For EP immunization, 87.5% of vaccinated birds seroconverted after the first vaccination and 100% seroconverted after the second vaccination and the H6 HI antibody titres were significantly higher than for chickens vaccinated by IM inoculation. Another group was given a single dose IM vaccination with 100 µg of the pCAG-HAk construct and showed a maximum sero-conversion rate of 53.3% with a peak H6 HI titre of 2^7 at 5 weeks post-vaccination. This demonstrated that optimization of the expression vector and insertion of a Kozak sequence could synergistically enhance expression of the H6 HA gene and result in a measurable H6 antibody response in SPF chickens. EP was also compared with IM inoculation with the 100 µg pCI-HA construct in SPF chickens, resulting in a 50% sero-conversion rate and mean HI titre of $2^{1.3}$ at 2 weeks after the second vaccination by EP. By comparison, only 25% chickens had trace HI titres by IM inoculation. This indicated that EP was more efficient than IM delivery for both constructs.

A codon-optimized complete HA gene from A/coot/WA/2727/79 (H6N2) was then chemically synthesized and cloned into a pCAGGS vector to generate the pCAG-

optiHAk construct. SPF chickens immunized twice with either 10 µg or 100 µg of pCAG-optHA showed 37.5% and 87.5% sero-conversion rates respectively, with a mean H6 HI tire of $2^{1.4}$ and $2^{2.6}$ at 3 weeks after the second immunization, but the differences were not statistically significant. There were also no significant differences in either the sero-conversion rate or the H6 HI titre between the pCAG-HAk and pCAG-optiHAk groups, suggesting that a codon-optimized HA DNA vaccine did not achieve significantly better immunogenicity than the pCAG-HAk vaccine.

In vitro expression of the developed DNA constructs in chicken-, hamster-, monkey- and human-origin cells, as measured by Western blotting and immunofluorescence testing (IFT), showed the strength of H6 HA expression in the following descending order - pCAG-optiHAk/pCAG-HAk, pCI-HAk, VR-HA, pCI-HA, pCIneo-HA and pVAX-HA. The *in vivo* chicken vaccinations also showed that the pCI-HA construct was more effective than the pCI-neo-HA, and that the pCAG-optiHA or pCAG-HAk constructs were better than pCI-HAk in term of reduction in virus shedding after H6N2 virus challenge. Thus, *in vitro* HA gene expression directly correlated with the generation of immune responses *in vivo*, indicating that *in vitro* studies can be used for pre-selection of expression plasmids prior to development of avian influenza DNA vaccines.

Lipofectin as a chemical adjuvant was shown to enhance the DNA-induced immune response but is prohibitively expensive for routine use in poultry vaccines. Thus, an experimental adjuvant for poultry DNA vaccines (Essai) and a new nanoparticle (Phema) adjuvant used for the first time in poultry were compared with conventional aluminum salts (alum) adjuvant in the present study. No HI antibody was detected in any adjuvant-vaccinated Hy-Line chickens following two immunizations. However, in comparison

with the naive control group, the alum- and Phema adjuvanted pCAG-HAk groups significantly reduced the frequency of virus shedding in oropharyngeal swabs, but Essai adjuvant was not effective in augmenting the pCAG-HAk vaccine efficacy. This pilot study also emphasised that the traditional aluminum hydroxide adjuvant, either DNA binding or non-binding, may be useful as an adjuvant for enhancing DNA-induced immune responses in chickens owing to its low price and safety record.

Overall, DNA immunization with various HA-expressing constructs was shown to be variably effective in inducing immune responses in chickens. The efficacy of DNA vaccines could be synergistically improved by taking appropriate approaches. With continuing research DNA vaccines have the potential to become an important tool for disease prevention and control.

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Abbreviations

AAHL	Australian Animal Health Laboratory
Ab	antibody
AH	aluminum hydroxide
AI	avian influenza
AIV	avian influenza virus
AIDS	acquired immune deficiency syndrome
alum	aluminum salts
Amp^r	ampicillin resistance
APC	antigen presenting cell
APS	ammonium persulfate
bp	base pair
BGH	bovine growth hormone
BHV-1	bovine herpesvirus 1
BSR	BSR cells are a clone of BHK-21 (baby hamster kidney cells)
cDNA	complementary DNA
CEF	chicken embryo fibroblasts
CMI	cell-mediated immune
CMV/IE	cytomegalovirus immediate early gene
CPE	cytopathogenic effect
CpG	cytosine-phosphate-guanine
CRBC	chicken red blood cell
CS	cloacal swabs
CT	cholera toxin
CTL	cytotoxic T lymphocyte
DAB	3, 3'-diaminobenzidine
DAFWA	Department of Agriculture and Food of Western Australia
DC	dendritic cell
DEPC	diethypyrocarbonate
dH_2O	distilled water

DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP)
dsDNA	double-stranded DNA
EB	ethidium bromide
E.coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
EID_{50}	egg-infective dose
ELISA	enzyme-linked immunosorbent assay
EP	electroporation/electropermeabilization
FCS	foetal calf serum
FITC	fluorescein isothiocynate
FMD	foot and mouth disease
gB and gD	glycoprotein B and glycoprotein D of herpesviruses
GG	gene gun
GMT	geometric mean titres
β-gal	β-galactosidase
HA	haemaggglutinin/haemagglutination
HA-DNA	plasmid encoding HA gene
HBSS	Hank's balanced salt solution
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HEPA	high efficiency particulate air
HI	haemagglutination inhibition
HIV	human immunodeficiency virus
HPAI	highly pathogenic avian influenza
HRP	horseradish peroxidase
HSV-1	herpes simplex virus 1
ID	intradermal
ID50	50% infectious dose
IFN	interferon

IFT	immunofluorescent test
IgG	immunoglobulin G
IL	interleukin
IM	intramuscular
IN	intranasal
IP	intraperitoneal
IPTG	isopropyl-β-thiogalactopyranoside
ISS	immunostimulatory sequences
IV	intraveneous
IVPI	intravenous pathogenicity index
IVV	inactivated virus vaccine
Kan^r	kanamycin resistance
LB	Luria-Bertani medium
LTR	long terminal repeat
LPAI	low pathogenic avian influenza
M	matrix protein
M-DNA	plasmid encoding M gene
mAb	monoclonal antibody
MDCK	Madin-Darby canine kidney cells
MCK	muscle-creatine kinase
MCS	multiple cloning site
MEGA	molecular evolution genetics analysis program
MHC	major histocompatibilty complex
MIDGE	minimalistic immunogenically defined gene expression
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
NA	neuraminidase
NA-DNA	NA-expressing plamid DNA
ND	Newcastle Disease
NDV	Newcastle Disease virus
NP	nucleoprotein

NP-DNA	plasmid encoding NP gene
NS1-DNA	plasmid encoding NS1 gene
Nt	nucleotide
OIE	the World Organisation for Animal Health
ORF	open reading frame
OD	optical density
ODNs	oligodeoxynucleotides
OS	oropharyngeal swabs
pCA	pCAGGS
POC	post-challenge
PEG	polyethylene glycol
PRC	prior to challenge
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBST	0.05% Tween-20 in PBS
PCR	polymerase chain reaction
PLG	poly-lactide-*co*-glycolide
PLGA	poly-DL-lactic-*co*-glycolic acid
PLG-CTAB	poly(lactide-*co*-glycolide)-cetyltrimethylammonium bromide
PR8	A/Puerto Rico/8/34 (H1N1)
RNA	ribonucleic acid
RSV	Rous sarcoma virus
RT	reverse transcriptase
SA	sialic acid
SAN	specific antibody negative
SC	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFV	Semliki forest virus
SHIV	simian-human immunodeficiency virus

SIN	Sindbis virus
SIV	simian immunodeficiency virus
SPF	specific pathogen-free
SV40	simian virus 40
TBS	Tris-buffered saline
$TCID_{50}$	50% tissue culture infective dose
TE	Tris-EDTA buffer
TEMED	N,N,N', N'-tetra methyl ethylene diamine
Th	T helper
TLR	Toll-like receptor
Tm	melting temperature of dsDNA
TNF	tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
VR	VR1012
VTM	viral transport medium
WB	Western blotting
W/O	water-in-oil
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of Unit Abbreviations

°C	degrees Celsius
µg	micrograms
µL	microlitre
µM	micromolar
µg/mL	microgram per milliliter
A450	absorbance at 450 nanometres
bp	base-pair
g	grams
g	times gravity
g/L	grams per litre
hr(s)	hour(s)
kb	kilobases
kDa	kilodalton
kV	kilovolts
M	molar
mg/mL	milligram per milliliter
L	litre
mg	milligrams
min	minutes
mL	millilitre
mm	millimetre
mM	millimolar
MW	molecular weight
ng	nanogram
nm	nanometres
OD	optical density
rpm	revolutions per minute
sec	seconds

U	units of penicillin or enzyme activity
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume

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Presentations

Oral Presentations

“Evaluation of DNA vaccines encoding avian influenza virus HA or NP gene in chickens”.Australian Biosecurity Cooperative Research Centre (AB-CRC) national workshop, Bangkok, Thailand, 2008

“Can DNA vaccine induce measurable antibody immune response to avian influenza virus in chickens?”. AB-CRC national workshop, Melbourne, Australia, 2007

“Biological properties of avian influenza virus A/coot/WA/2727/79(H6N2)”.
AB-CRC national workshop, Cains, Australia, 2006

“Development and immunological evaluation of novel DNA vaccines against avian influenza”. Postgraduate Seminar Programme, School of Veterinary and Biomedical Siences (VBS), Murdoch University, 2006

Poster Presentations

“Development of a nano-vaccine against a wild bird H6N2 avian influenza virus” .
Vaccine Third Gobal Congress, October, Singapore, 2009

“Comparison of three DNA expression vectors encoding HA gene of H6N2 avian influenza virus in chickens”. Postgraduate Poster Day, VBS, Murdoch University, 2008

“Can DNA vaccine induce measurable antibody immune response to avian influenza virus in chickens?”. Postgraduate Poster Day, VBS, Murdoch University, 2007

“Development and immunological evaluation of a novel DNA vaccine against avian influenza”. Postgraduate Poster Day, VBS, Murdoch University, 2006

“Development of a new strategic vaccine for influenza control”. Postgraduate Poster Day. VBS, Murdoch University, 2005

Chapter 1
Introduction and Literature Review

1.1 Introduction

Avian influenza (AI) is a disease of birds, and occasionally other mammals and humans, caused by any type A avian influenza virus (Easterday and Hinshaw 1991). Although listed as a notifiable disease by the World Organization for Animal Health (OIE), the majority of AI viruses do not cause significant diseases in birds (OIE 2008). The very virulent AI viruses, generally restricted to subtypes H5 and H7, can cause highly pathogenic avian influenza (HPAI) which was first described in 1959 in Scotland due to A/chicken/Scotland/59 (H5N1) (Alexander 2000; Pereira *et al.* 1965).

Since late 2003, the apparently unprecedented emergence and worldwide spread of the H5N1 HPAI virus and resultant fears of a pandemic outbreak have brought AI to the forefront of important animal diseases (Alexander 2007). Vaccination is a powerful and cost-effective tool to combat AI for either emergency or prophylactic purposes in large scale outbreaks (Capua and Marangon 2007b). Although successful in prevention and control of avian influenza epizootics, traditional protein-based vaccines such as whole, inactivated or live, recombinant vectored virus vaccines have some inherent limitations. These include safety issues due to use of live virus in vaccine production, variable immunity due to vector immunity for live recombinant vaccine vectors, and a heavy reliance on chicken embryonated eggs for vaccine production (Ben-Yedidia and Arnon 2005; Capua and Marangon 2007a; Greenland and Letvin 2007; Leitner *et al.* 1999). With the difficulties in controlling the current H5N1 epizootic in multiple countries using

existing strategies, including vaccination, there is a clear need to examine alternative vaccine strategies.

Genetic immunization, also referred to as DNA or polynucleotide immunization, represents a novel strategy for vaccine development, in which plasmid DNA encoding foreign antigens is directly administered to a host and leads to induction of a specific immune response to the *in vivo* produced antigens (Cohen *et al.* 1998). In contrast to currently used vaccines, immunization with DNA confers a cytotoxic T lymphocyte (CTL) response via the major histocompatibility complex (MHC) class I-restricted pathway, and a humoral response, as well as a helper T lymphocyte (Th) response via MHC class II-restricted pathway (Donnelly *et al.* 2005). DNA vaccines are likely to be attractive owing to reduced safety concerns in comparison with the use of live viruses in conventional vaccine production, expeditious vaccine development and production, relative ease of manufacture in large quantities, and more robustness regarding transportation and storage requirements (Donnelly *et al.* 1997b; Gurunathan *et al.* 2000; Lalor *et al.* 2008). Thus, DNA vaccines have the potential to address many of the shortcomings of current vaccines and show great potential for application to large scale vaccination for avian influenza.

DNA vaccines have been developed to protect against a number of pathogens in a range of animals (Donnelly *et al.* 1997b; Dufour 2001; Liu and Ulmer 2005). DNA vaccines encoding different genes of influenza type A viruses have been demonstrated to generate variable immune responses in several different species, including mice (Epstein *et al.* 2002; Kodihalli *et al.* 2000) , chickens (Kodihalli *et al.* 1997; Robinson *et al.* 1993), swine (Heinen *et al.* 2002; Macklin *et al.* 1998), ferrets (Ljungberg *et al.* 2002; Webster

et al. 1994), and nonhuman primates (Liu *et al.* 1997). However, the mechanism of DNA vaccines has not yet been fully elucidated. Low immunogenicity and inter-individual variability are considered to be the main bottlenecks for development of commercial DNA vaccines (Mir *et al.* 1999) . In this study a comprehensive study has been conducted for the first time to develop DNA vaccines against an LPAI virus (H6N2) as opposed to DNA vaccines against HPAI viruses, to investigate if this can provide information on immunogenicity and variability in DNA vaccines.

Studies on a DNA vaccine encoding the M2 protein of influenza virus showed contradictory results in a mouse or swine model, emphasizing the need to evaluate vaccine efficacy in appropriate animal models (Heinen *et al.* 2002). It is believed that DNA vaccines show success in mouse models but are unsatisfactory in larger animals and humans (Abdulhaqq and Weiner 2008). This experience emphasizes the need to choose the appropriate animal models for evaluation of vaccine efficacy (Kemble and Greenberg 2003; Kodihalli *et al.* 2000). In this thesis the chicken, a natural host for avian influenza and one in which DNA vaccines may be used, was chosen to assess the efficacy of AI DNA vaccines that were developed.

For Australian biosecurity reasons, the virus selected for the development of an avian influenza DNA vaccine was restricted to a low pathogenic avian influenza (LPAI) virus as any research relating to HPAI viruses is restricted to the Australian Animal Health Laboratory (AAHL), CSIRO, Geelong, Australia. Avian influenza virus A/Eurasian coot/Western Australia/2727/1979 (H6N2) (A/coot/WA/2727/79) was isolated from a healthy eurasian coot (*Fulica atra*) in Western Australia in the 1970's, as part of an international program on the ecology of influenza in Australia supported by the World

Health Organization (WHO) (Mackenzie *et al.* 1984). Thus, this study was conducted as a proof-of-concept project for an avian influenza DNA vaccine in chickens.

Initial characterization of the virus was performed at the Virology Laboratory, Department of Agriculture and Food of Western Australia (DAFWA). Development of several DNA vaccines using different vectors and adjuvants was conducted at the the State Agricultural Biotechnology Centre (SABC) at the Murdoch University. Vaccine evaluation studies were conducted mainly in the Animal Houses and Animal Isolation House at Murdoch University and partially (Chapter 3) at an AEC-approved free-range pen at Jandakot, Western Australia with approval of the Animal Ethics Committee (AEC) and Institutional Biosafety Committee at Murdoch University. The chickens used were supplied by Altona Hatchery Pty. Ltd., Australia. The flock of origin was avian influenza free and the chickens were influenza A antibody negative.

The study relating to use of an additional pCAGGS vector and codon optimization of the HA gene (Chapter 5 and 6) was conducted at the Avian Influenza Reference Laboratory at Harbin Veterinary Research Institute (HVRI), China under a placement studentship from the Australian Biosecurity Cooperative Research Centre (AB-CRC).

1.2 Avian influenza virus

1.2.1 Virus structure, genome and classification

Avian influenza is caused by avian influenza virus (AIV), which belongs to the genus influenzavirus A in the family Orthomyxoviridae (Easterday and Hinshaw 1991). Influenza viruses are medium-sized (80-120 nm in diameter), pleomorphic RNA viruses with a host-derived lipid bilayer envelope covered with about 500 projecting glycoprotein

spikes with haemagglutinating and neuraminidase activities (OIE 2008; Perez *et al.* 2005). The viral genome consists of eight segments of single-stranded, negative sense RNA, totaling approximately 14 kilobases, and encodes for 11 viral proteins (Chen *et al.* 2004; McGeoch *et al.* 1976) (Figure 1.1). Segments 1, 2 and 3, about half of the total genome, encode for the three viral polymerases (PA, PB1and PB2). An alternative open reading frame (ORF) near the 5' end of the PB1 gene encodes for the 90-amino acid-long PB1-F2 polypeptide, which has apoptotic or pro-apoptotic properties and may contribute to the pathogenicity and lethality of influenza type A viruses (Chen *et al.* 2004). Segment 5 encodes the nucleoprotein (NP). Segments 4 and 6 encode for the two external glycoproteins, haemaggglutinin (HA) and neuraminidase (NA), respectively. The two smallest segments (7 and 8) encode two genes each with overlapping reading frames. Segment 7 encodes two matrix proteins: M1, the most abundant protein in the influenza virion, forms a shell surrounding the virion nucleocapsids and initiates progeny virus assembly and M2, that has ion channel activity, is embedded in the viral envelope. Segment 8 encodes for a nonstructural protein (NS1) that is the only protein which is not packaged into the virus particle and which blocks the host's antiviral response, and NS2 or NEP (nuclear export protein) that participates in the assembly of virus particles (Palese and Shaw 2007; Steinhauer and Skehel 2002; Tamura *et al.* 2005; Webster *et al.* 1992).

The HA protein is a glycosylated integral membrane protein presented in a homotrimer form on the surface of the virus, which mediates adsorption and penetration of virus during infection. The protein NA is an integral membrane glycoprotein in a homotetramer form that promotes the release of virus particles from host cell receptors (Palese and Shaw 2007; Suarez and Schultz-Cherry 2000b). Two surface glycoproteins,

HA and NA, undergo gradual, continuous minor antigenic changes due to point mutations in the HA and NA genes, referred to as antigenic drift. This phenomenon occurs in all influenza A viruses due to the lack of a proof reading system for the RNA polymerases. Owing to the segmented nature of the viral genome, the exchange of RNA segments, called genetic reassortment, between two genotypically different AI viruses infecting the same host cell may potentially result in the generation of a novel strain and/or subtype, which can lead to major antigenic changes in the HA or NA genes. This is referred to as antigenic shift. The emergence of these resultant virus strains with different antigenic and other characteristics, including enhanced human infectivity, may result in influenza pandemics (Easterday and Hinshaw 1991; Palese and Shaw 2007; Tamura *et al.* 2005).

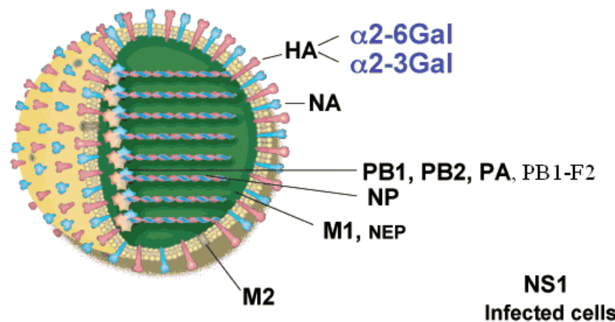

Figure 1.1 Schematic diagram of an influenza A virus.
(Modified from http://avianflu.umd.edu/Avian_Influenza_Program/Avianflu.html)

Influenza viruses are divided into types A, B, or C on the basis of the antigenic nature of M1 and NP proteins (Suarez and Schultz-Cherry 2000b). Type B and C viruses generally only infect humans, but the type A viruses infect humans, pigs, horses, mink, felids, marine mammals, and a wide variety of domesticated and wild birds (Olsen *et al.* 2006;

Tamura *et al.* 2005). Type A viruses are further subdivided into subtypes based on the antigenic differences in the HA and NA molecules. At present, there are 16 haemagglutinin (H1–H16) and 9 neuraminidase (N1–N9) subtypes. Each virus has one H and one N antigen subtype. All H and N subtypes of influenza A virus in the majority of possible combinations have been isolated from avian species (Alexander 2000; Palese and Shaw 2007; Tamura *et al.* 2005).

1.2.2 Avian influenza biotypes

Avian influenza viruses can be subdivided into two biotypes, designated low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) (Werner and Harder 2006). Infection of poultry with LPAI viruses can be associated with a spectrum of clinical signs from asymptomatic infections, mild to severe respiratory disease, growth rate and egg production losses. Infection of susceptible poultry with HPAI viruses, previously referred to as ‘fowl plague', can cause severe disease with high morbidity and mortality. Virus biotypes are determined by pathogenicity tests, e.g. intravenous pathogenicity index (IVPI), with strains considered to be highly pathogenic if the inoculation of a minimum of eight susceptible 4–8-week-old chickens with index virus strains causes more than 75% mortality within 10 days or has an IVPI of greater than 1.2. To date, HPAI viruses have only been associated with the H5 and H7 subtypes exhibiting multiple basic amino acids at the cleavage site of the HA molecules. This molecular characteristic of the HA provides an accurate indicator of virulence or potential virulence as multiple basic amino acid motifs at the cleavage site facilitate cleavage by weak proteases such as furin, whereas, the LPAI viruses require strong proteases like trypsin found in the respiratory and gastrointestinal mucosa. Most H5 or H7 isolates of low

virulence show the amino acid motif at the cleavage site of either -PEIPKGR*GLF- or -PENPKGR*GLF- (Alexander 2008; OIE 2008). Since 2004, due to the risk of a H5 or H7 virus of low virulence becoming virulent by mutation in poultry, all H5 and H7 viruses are now designated as notifiable avian influenza (NAI) viruses. NAI viruses are now further divided into highly pathogenic notifiable avian influenza (HPNAI) and low pathogenic notifiable avian influenza (LPNAI) according to the HA0 cleavage site amino acid sequence and strain virulence for chickens by pathogenicity tests. Non-H5 or non-H7 AI viruses which are not highly pathogenic in chickens are classified as LPAI (Alexander 2000; Capua and Alexander 2004b; OIE 2008) .

To date all true HPAI are of H5 or H7 subtypes. However, at least two isolates, both of H10 subtype (H10N4 and H10N5) were classified as HPAI due to IVPI values >1.2 according to both the OIE and EU definitions, whereas, they did not have multiple basic amino acids at the HA cleavage sites (Wood *et al.* 1996). These viruses caused no death or signs of disease when inoculated intranasally into chickens. On the contrary, four viruses containing multiple basic amino acids at the HA cleavage sites showed low virulence (IVPI <1.2) (Londt *et al.* 2007). Other unusual strains are H7N3 HPAI viruses (IVPI>1.2) isolated in Chile in 2002 (Suarez *et al.* 2004) and in Canada in 2004 (Pasick *et al.* 2005), which show the distinct and unusual cleavage site motif of PEKPKTCSPLSRCRETR*GLF and PENPKQAYRKRMTR*GLF, respectively. This is attributable to intragenic recombination between the HA, NP and M genes to give a virus with easily cleaved HA (OIE 2008).

1.2.3 Viral antigens involved in immunity

At least four out of 11 viral proteins are important targets of the host immune response. Both HA and NA are the most important protective components among the viral proteins. In terms of protective immune responses, HA and NA readily induce antibody responses whereas NP elicits cell-mediated immunity (Sasaki *et al.* 2004; Suarez and Schultz-Cherry 2000b; Tamura *et al.* 2005). Anti-HA antibodies block virus attachment to sialic acid receptors of the host cells and prevent infection, whereas, anti-NA antibodies bind viral NA glycoprotein at the cell surface to prevent the release of new virions from infected cells and hence accelerate the recovery from infection. Thus, the antibodies against HA and NA constitute the primary defence against homologous virus infection. In particular, antibody against the one virus is capable of neutralizing other viruses of the same HA or NA subtype, but does not cross-neutralize viruses of different HA and NA subtypes (Subbarao *et al.* 2006; Tamura *et al.* 2005). It is well accepted that these neutralizing antibodies produce immunological pressure and thus promote antigenic drift and shift in HA and NA proteins of influenza A viruses (Kodihalli *et al.* 1994).

Antibody to HA is the main determinant for protection of the host from influenza virus infection. In poultry, vaccination for avian influenza is targeted primarily at the HA subtype, which has been demonstrated by subunit vaccines that only contain the HA protein (Swayne *et al.* 2000a). NA protein can also stimulate production of neutralizing antibody and NA-specific vaccines provided protection against a HPAI challenge in chickens (McNulty *et al.* 1986). It is thought that the NA protein is less important than HA protein in protection (Johansson *et al.* 1989; Suarez and Schultz-Cherry 2000b; Tamura *et al.* 2005). However, the administration to mice of a plasmid DNA mixture encoding HA or NA genes by gene gun (GG) provided almost complete protection

against subsequent influenza A/Puerto Rico/8/34 (H1N1) (PR8) virus challenge, accompanied by high levels of specific antibody responses to the respective components (Chen *et al.* 1999a).

In contrast to the highly variable HA and NA proteins, the internal NP, M1 and M2 proteins show little antigenic variability within influenza type A viruses. These proteins are potential candidate antigens for development of vaccines that might provide cell mediated immunity and that can give broad cross protection against multiple HA and NA subtypes (Kodihalli *et al.* 1994; Tamura *et al.* 2005). Antibodies to conserved NP and M1 antigens have been utilised to detect type-specific antibody using agar gel immunodiffusion (AGID) and Enzyme-linked immunosorbent assay (ELISA) in chickens (Suarez and Schultz-Cherry 2000b). However, for a vaccine to prevent AI virus infection, NP and M1 antigens are not effective as antibodies to these proteins are not neutralizing and hence are not protective. The M2 protein has provided broad but limited cross-protection in mice. This has not been demonstrated in chickens yet (Lalor *et al.* 2008; Suarez and Schultz-Cherry 2000b).

1.2.4 General features of avian influenza virus epidemiology

Influenza A viruses have been shown to infect a wide variety of birds and mammals. Wild waterfowl, gulls and shorebirds are thought to form the virus reservoir in nature (Alexander 2007; Horimoto and Kawaoka 2005; Suarez 2000; Webster *et al.* 1992). LPAI viruses have thus far been isolated from at least 105 wild bird species of 26 families (Olsen *et al.* 2006). The actual number of susceptible species is possibly much greater (Alexander 2007). Influenza A virus-infected birds, showing no clinical signs, can transmit these viruses to other avian or mammalian populations and consequently may

seed these viruses to new geographic areas. If these LPAI viruses are introduced into a domestic poultry flock, most are either non-pathogenic or mildly pathogenic for poultry. However, some LPAI H5 and H7 isolates may undergo rapid spread in susceptible poultry and in this process progressively incorporate mutations giving multiple basic amino acids at the HA cleavage site or other virulence characteristics, becoming highly pathogenic and causing HPAI outbreaks (Alexander 2007; Normile 2005; Olsen *et al.* 2006). It is believed that the wider the circulation of LPAI in poultry, the higher the posssiblity of mutation to HPAI (Alexander 2007). This is particularly so for H5 or H7 subtype viruses (Garcia *et al.* 1996).

The mechanisms by which influenza viruses are transmitted from one bird to another and cause infection are poorly understood, but this appears to depend on the strain of virus, the species of bird, and environmental factors (Alexander 2007). Historically, HPAI was regarded as a rare disease in domestic poultry with only 17 outbreaks recorded worldwide, five in turkeys and 12 in chickens, during the period from 1959 to 1998 (Alexander 2000; Capua and Alexander 2004a). However, there were a further eight HPAI outbreaks involving 12 countries from 1997 to March 2004 (Morris and Jackson 2005). Since late 2003, there has been a dramatic increase in the number of outbreaks (a total of 2544 H5N1 HPAI outbreaks from the end of 2003 to 15 July 2009) and infections have spread widely from Southeast Asia to other Asian countries, the Middle East, Europe and Africa, so that now over 50 countries have been affected (Lalor *et al.* 2008; OIE 2009). In addition to affecting land-based poultry, recent outbreaks have involved deaths in ducks, geese and a range of wild bird species as well as some feline species (Poland *et al.* 2007). In terms of the number of infected flocks and the geographical spread of the diseases, this

H5N1 virus lineage has caused the largest HPAI epizootic ever seen in the world (Sims *et al.* 2005; Stephenson 2006). This has not only resulted in tremendous losses to the world poultry industries and caused substantial loss of food resources in developing ountries, but as the virus has spread directly to humans and caused often-fatal infections, this has created fears of a devastating human pandemic outbreak with high mortality.

Prior to 1997, it was believed that the avian influenza viruses were unable to be transmitted directly to humans due to the absence of an appropriate attachment site on the HA to bind to human cellular receptors (Beare and Webster 1991; Kodihalli *et al.* 1999). However, a novel H5N1avian influenza virus was transmitted directly from infected poultry to humans in Hong Kong in 1997, causing 18 confirmed infections and six deaths. This provided the first clear evidence that AI viruses could pass directly to humans (Claas *et al.* 1998; Subbarao *et al.* 1998). This virus disappeared after the total depopulation of poultry in Hong Kong in 1997. However, since then there have been further cases where human infections have resulted from contact with poultry during avian influenza outbreaks, caused by AI virus subtypes including H5N1 (Capua and Alexander 2004a), H7N3 (Tweed *et al.* 2004), H7N7 (Fouchier *et al.* 2004) , H9N2 (Peiris *et al.* 1999) and H10N7 (PAHO 2004). From 2003 to mid-2009 these viruses have infected at least 417 humans in 15 countries and caused 257 deaths (WHO 2009).

1.2.5 Prevention and control of avian influenza

With this ongoing H5N1 HPAI panzootic in poultry and the risk of an H5N1 pandemic, a comprehensive multi-faceted control strategy needs to be undertaken. This strategy is similar to control programs used for control of other HPAI outbreaks. However, with the rapid onset of geographically diverse outbreaks in countries with variable animal health

resources during this H5N1 panzootic, the OIE/FAO/WHO has recommended the following components: (a) enhanced biosecurity, (b) increased surveillance with effective diagnostic tests, (c) early detection and rapid confirmation of suspects, (d) rapid and transparent notification, (e) quarantine (including containment, management of poultry movement, zoning and compartmentalization), (f) stamping-out of outbreak cases, (g) vaccination, (h) other measures such as education, communication and mass media campaigns to increase public awareness and reduce panic and education of farmers and poultry workers on avian influenza control measures (FAO/WHO/OIE 2007; Swayne 2006, 2009). Stamping out and depopulation plus enhanced biosecurity measures seems to be the most effective means of eradication of HPAI and this has worked in developed countries in previous H5 and H7 outbreaks and is probably the most effective method for limited outbreaks in non-densely populated poultry areas. However, stamping-out policies cannot be adopted in developing and undeveloped countries for logistical reasons, including loss of food resource, damage to the microeconomy and lack of funds to conduct campaigns and compensate poultry owners (Capua and Alexander 2004a).

Vaccination has proven to be a powerful tool for control of H5N1 HPAI outbreaks, supporting eradication programs through increasing bird resistance to field virus challenge, reducing virus shedding levels in vaccinated birds and hence preventing virus transmission (Capua and Alexander 2004a; Capua and Marangon 2007b; van der Goot *et al.* 2005). Vaccination and companion DIVA ("differentiation infected from vaccinated animals") testing, as well as quarantine and movement control are highly recommended by OIE for control and prevention of HPAI (Capua and Marangon 2007a).

The origins for the H5N1 panzootic are unknown. Wild birds and waterfowls as reservoirs play a potential role in AIV circulation and evolution (Normile 2005; Olsen *et al.* 2006). However, the global distribution and persistence of LPAI viruses in wild bird populations is not fully elucidated and is often difficult to get data on (Webby and Webster 2003). With lack of knowledge of the sheer magnitude of the animal reservoirs, it is impossible to predict if and when outbreaks will occur (Alexander 2007). Furthermore, influenza viruses can have numerous antigenic subtypes and rapidly evolve due to constant gene mutation and reassortment. All these factors contribute to the fact that AI is a difficult disease to eradicate in some circumstances. Vaccination has emerged as a useful tool in managing H5N1 HPAI infections with a view to future eradication (Webster *et al.* 1992; Webster *et al.* 2006).

1.3 Vaccination against avian influenza and challenges with vaccination

Vaccines have been widely used as a valuable tool to prevent, manage or eradicate AI from poultry or other birds in various countries (Swayne 2009). A wide variety of vaccines have been developed in the laboratory for potential use in the field (Kemble and Greenberg 2003; Swayne 2006). However, there is no globally-effective or universal AI vaccine due to continuous mutations or genetic reassortment of these viruses (Swayne 2006).

1.3.1 Inactivated whole virus vaccine

As early as 1971, inactivated oil emulsion vaccines were used to immunize chickens and turkeys against AI virus infection (Allan *et al.* 1971). Up to now, inactivated vaccines against AI in chickens and turkeys have been reported against AI subtypes H1 (Swayne

2001), H4 (Abraham *et al.* 1988; Fatunmbi *et al.* 1992), H5 (Crawford *et al.* 1998; Ellis *et al.* 2004; Fatunmbi *et al.* 1992; Swayne *et al.* 2001; Swayne *et al.* 2006), H6 (Cardona *et al.* 2006), H7 (Cherbonnel *et al.* 2003; Di Trani *et al.* 2003; Fatunmbi *et al.* 1992; Philippa *et al.* 2005) and H9 (Pan *et al.* 2009; Swayne 2006).

Currently, inactivated whole virus vaccines are the major vaccine type used in the poultry industry. They engender a strong humoral immune response and have proven to be effective against AI in multiple species of poultry. Nevertheless, they don't produce a strong mucosal immune response. In addition, inactivated oil emulsion vaccines elicit antibodies not only specific for the protective epitopes on the HA and NA, but also to the internal proteins, NP and M1. Thus, vaccinated birds cannot be differentiated from naturally infected birds using the commonly used serologic assays like influenza A ELISA or AGID tests (Marangon *et al.* 2007; Suarez 2005).

Reverse genetics have been applied to develop improved vaccines against AI (Liu *et al.* 2003; Neumann *et al.* 2003; Tian *et al.* 2005; Webster *et al.* 2006). Conventionally prepared reassortant H5N1 vaccines developed by reverse genetics are currently being used in China (Tian *et al.* 2005) and other countries in SE Asia (Liu *et al.* 2003; Swayne 2009). In addition, H7 and H9 subtype vaccines developed by reverse genetics have been used in experimental challenge studies (Chen *et al.* 2003b; Joseph *et al.* 2008). These vaccines have similar performances to conventional inactivated vaccines, however extensive data on their efficacy under field conditions is not available. In addition, this technology advancement does not alter the fact that large scale vaccine production still relies on egg-based production. This process is cumbersome, lengthy, and costly (Swayne 2009; Wang *et al.* 2006).

1.3.2 Live recombinant vectored based vaccine

Protective viral antigens can be expressed *in vivo* by inoculation of live viral vectors containing the gene for the target antigen. These vaccines have been developed for poultry using viral vectors such as fowl poxvirus (Swayne *et al.* 2000a), vaccinia virus (De *et al.* 1988; Yewdell *et al.* 1985), retrovirus (Brown *et al.* 1992; Hunt *et al.* 1988), adenovirus (Gao *et al.* 2006; Hoelscher *et al.* 2006; Toro *et al.* 2007), Newcastle disease (ND) virus (Ge *et al.* 2007; Park *et al.* 2006; Swayne *et al.* 2003; Veits *et al.* 2006), infectious laryngotracheitis herpesvirus (Veits *et al.* 2003) and an avian influenza-Newcastle disease virus chimera (Park *et al.* 2006).

Among these vaccines, a recombinant fowlpox virus vectored vaccine expressing influenza H5 HA has been most commonly used and is commercially available (Suarez and Schultz-Cherry 2000b; Swayne 2009). Recombinant fowlpox vectors expressing HA genes from A/turkey/Ireland /1378/83 (H5N3) (Swayne *et al.* 2000a), A/seal/Mass/1/80 (H7N7) and A/chicken/Victoria/1/85 (H7N7) (Boyle *et al.* 2000), and A/turkey/Ireland/1378/83 (H5N8) (Bublot *et al.* 2006) have been reported to be effective in reducing virus shedding and in providing clinical protection. Recently, a new recombinant vaccine co-expressing both HA (H5) and NA (N1) genes of A/goose/Guangdong/3/96 (H5N1) was developed (Qiao *et al.* 2006; Qiao *et al.* 2003). This vaccine is suitable for use in 1-day-old chickens and in older birds where no antibody to fowlpox virus exists. Although any test developed to detect antibodies to the virus NP, M, NS1 or NA protein can be used to identify field exposed birds in a fowlpox-vectored vaccinated population, there is some uncertainty about the efficacy of fowlpox-vectored vaccines, relating to the immune status of flocks to the vector virus (Swayne *et*

al. 2000a). Thus, these vaccines are likely to be used only in birds that are susceptible to infection with the vector virus (Capua and Marangon 2006; Swayne *et al.* 2000a). Moreover, some studies with these vaccines appear to have shown evidence of a period of growth inhibition in chickens (Mingxiao *et al.* 2006).

Most recently, recombinant Newcastle Disease virus (NDV) vectors expressing HA of H5 or H7 HPAI virus, developed using reverse genetics, have been licensed for use in China and Mexico (Swayne 2009). This prototype vaccine not only elicited both NDV- and AIV-specific antibodies, but also protected chickens from AIV and NDV challenge (Ge *et al.* 2007; Park *et al.* 2006). However, pre-existing maternal and active humoral immunity to ND virus vector or AI virus may limit vaccine replication and thus prevent or reduce a protective immune response. Additionally, biosafety and biosecurity of such live vaccines has yet to be fully assessed (Swayne 2009).

1.3.3 Subunit avian influenza vaccines

Viral proteins such as the AI HA can also be expressed in an *in vitro* system and this crude or purified viral protein can be formulated with adjuvant to form a subunit vaccine. This makes the vaccine technically similar to inactivated whole AI vaccines, but production of viral antigens does not involve culture of live AIVs and avoids biosecurity concerns. *In vitro* expression systems include eukaryotic cell cultures (such as mammalian cells (Ghendon *et al.* 2005), plants (Shoji *et al.* 2008), yeast (Saelens *et al.* 1999), bacterial (e.g. *Escherichia coli* (Davis *et al.* 1983)) or viral vectors (e.g. baculovirus) (Crawford *et al.* 1999; Johansson 1999; Swayne *et al.* 2001). Recently, *Pichia pastoris*, one of the eukaryotic expression systems, was used to express HA genes from H5, H7 and H9 AI viruses respectively (Xu *et al.* 2006). The production costs of

this type of vaccine may not be compatible with poultry use where cost/dose must be substantially lower than human or some other animal vaccines (Suarez and Schultz-Cherry 2000b).

1.3. 4 Genetic vaccines

Genetic vaccines are composed only of DNA (as plasmids) or RNA (as mRNA), which is taken up by cells and translated into protein (Leitner *et al.* 1999). Since there are limited reports on RNA vaccines compared to extensive literature on DNA vaccines, genetic vaccines generally refer to plasmid DNA antigen-expression systems (Davis 1997). Genetic immunization, also termed DNA or polynucleotide immunization, is a novel vaccine technology that developed following the first report that direct injection of plasmid DNA generated the successful expression of the plasmid-encoded antigen in murine muscle cells (Wolff *et al.* 1990). This unexpectedly successful new method has been described as the "Third Vaccine Revolution" (Babiuk *et al.* 1999a; Dixon 1995) or "the third generation of vaccines" (Pasquini *et al.* 1997).

1.3.4.1 DNA-based vaccines

DNA vaccines have been proven to be effective against various infectious diseases (Deck *et al.* 1997; Donnelly *et al.* 1997b; Dufour 2001). Numerous studies of influenza A DNA vaccines have demonstrated that they are capable of inducing humoral and cell mediated immune responses and conferred protection against influenza A viruses in a range of species via various delivery routes (Babiuk *et al.* 1999b). However, DNA vaccines against AI in poultry have not been as efficacious and consistent as conventional inactivated whole virus vaccines (Suarez and Schultz-Cherry 2000a).

1.3.4.2 RNA-based vaccines

To avoid the potential risk of DNA sequence integration into the host genome with DNA vaccination, RNA has been proposed as the expression vector (Vignuzzi *et al.* 2001). The mRNA constructs encoding the foreign gene, delivered either intramuscularly (Conry *et al.* 1995), by GG (Qiu *et al.* 1996), or by liposome-entrapped mRNA injection (Martinon *et al.* 1993), resulted in efficient transgene expression and induced immune responses. However, antigen expression sufficient to stimulate effective immune responses has been a major problem with this type of vaccine.

1.3.5 Other AI vaccines

1.3.5.1 Live attenuated influenza virus vaccines

A reassortant H9N2 vaccine candidate carrying HA and NA genes from A/chicken/Hong Kong/G9/97 (H9N2) virus (Chen *et al.* 2003b) and a reassortant H5N1 influenza A virus vaccine candidate bearing the HA and NA genes of the A/HK/491/97 (H5N1) virus (Subbarao *et al*. 2003) in a background of internal genes from PR8 virus, were developed using reverse genetics and showed ability to induce protective antibody responses in experimental studies. As the vaccine virus is live, there is potential for reassortment with other circulating influenza A viruses with the risk of novel influenza viruses. To date, no genetically engineered live AI virus vaccines are commercially available for use in animals. However, cold-adapted temperature sensitive mutant influenza A virus vaccines, derived from the cold-adapted (ca) influenza A vaccine donor strain influenza A/Ann Arbor/6/60 (H2N2), have been developed by reverse genetics and are safe for use in

humans (Chen *et al.* 2003a; Suguitan *et al.* 2006). Potentially, a live AI virus vaccine could be developed for use in poultry if sufficient safety was demonstrated.

1.3.5.2 Non-parenteral administration of inactivated whole vaccines

Mucosal vaccination using existing inactivated whole influenza A virus vaccines induced both mucosal and systemic antibody responses and conferred broad cross-protection in mice (Takada *et al.* 1999; Takada *et al.* 2003; Tumpey *et al.* 2001). Oral immunization with an inactivated waterfowl-origin AI virus has been shown to induce specific antibody to this virus, indicating that it should at least be possible to use oral influenza vaccination in poultry (Crawford *et al.* 1998).

Altogether, although different kinds of vaccines were experimentally and commercially developed for use in the poultry industry, each vaccine seems to have its limitations. To date, licensed vaccines, either inactivated whole virus vaccines or recombinant live fowlpox or NDV-based vaccines, rely on embryonated chicken egg production. This leads to some drawbacks for large scale vaccine production, including strict requirement for facilities of a high biosecurity standard for handling live influenza viruses, long production cycles, heavy reliance on manual systems for production, the need for readily available chicken flocks, inflexibility in quickly altering antigenic composition and limited breadth of protection (Dufour 2001; Forde 2005; Rao *et al.* 2009). Examining alternative strategies is necessary for development of improved or more cost-effective vaccines.

1.4 Characteristics of DNA-based vaccines

DNA vaccines are composed of simple ring forms of double-stranded DNA that generally consist of two components: (1) a mammalian expression cassette (promoter/enhancer, antigen-encoding DNA and polyadenylation sequences) and (2) the bacterial plasmid backbone (necessary for plasmid amplification and selection) (Gurunathan *et al.* 2000; Miller *et al.* 2004; Srivastava and Liu 2003). Since its initial development in 1990, this technology has been used to generate humoral and cell-mediated immune responses in a wide variety of species for numerous viral (Davis and McCluskie 1999), bacterial (Strugnell *et al.* 1997) and parasitic (Kalinna 1997) diseases in a range of animal models (Donnelly *et al.* 1997b; Dufour 2001; Ivory and Chadee 2004). They have also been used for genetic therapies against cancer (Benton and Kennedy 1998) and autoimmune diseases (Ramshaw *et al.* 1997). In addition, DNA vaccines have become a valuable laboratory tool for a variety of applications ranging from proteomics to understanding the mechanism of antigen presentation, the role of cytokines and the effects of bacterial DNA in the generation of immune responses (Donnelly *et al.* 1997b).

DNA vaccines are prepared using standard molecular biology techniques. First, a gene of interest is cloned into a mammalian expression vector using polymerase chain reaction (PCR), with a pair of primers and a cDNA template. Secondly, the resultant plasmid construct is examined to verify the fidelity of the insert to avoid cloning errors, such as frame shifts through sequencing. Following successful sequence analysis, the construct is tested to verify correct protein expression by a variety of methods. Generally a cell line is transfected transiently with the plasmid and the desired protein expression is detected by

Western blotting (WB), ELISA, immunofluorescent test (IFT), or immunoprecipitation. Finally, the construct is grown and purified for immunization (Sasaki *et al.* 2003).

1.4.1 Immunology of DNA vaccines

1.4.1.1 Immune mechanism

Although the ability of DNA vaccines to induce immune responses has been well documented, the mechanism by which the immune cascade arises is not yet fully understood. Following delivery by various techniques via numerous routes, plasmid DNA is taken up primarily by somatic cells (keratinocytes for GG and myocyte for intramuscular (IM) injection) (Dupuis *et al.* 2000; O'Hagan *et al.* 2004) and to some extent by antigen presenting cells (APCs), and the DNA encoded vaccine antigen is then expressed (Danko *et al.* 1997; Donnelly *et al.* 1997b; Klinman *et al.* 1998; Lu *et al.* 1996; O'Hagan *et al.* 2001; Wolff *et al.* 1990). Endogenously synthesized proteins produced by transfected cells after plasmid DNA inoculations are processed in the cytosol by multicatalytic proteosomes and resultant peptides are presented via the MHC Class I pathway to activate $CD8^+$ T lymphocytes (Cohen *et al.* 1998; Tuting *et al.* 2000). Soluble proteins released by transfected cells undergo endocytosis or phagocytosis to activate the MHC class II-restricted pathway for antigen processing in $CD4^+$ T cells, which can promote antibody responses in B cells or initiate other T helper (Th) cell responses (Cohen *et al.* 1998; Gurunathan *et al.* 2000; Tuting *et al.* 2000). T helper cells, also known as effector T cells, secrete different and sometimes mutually antagonistic cytokines, which are fundamental for the complete activation of either B cells (Th2-type) or CTLs (Th1-type) (Davis 1997). Since the quantity of endogenously synthesized antigen was demonstrated to range from picograms to nanograms, the efficient induction

of a broad-based immune response most likely involves the immune-enhancing properties of the plasmid DNA itself (e.g. CpG motifs) and/or the type of antigen-presenting cell (APC) transfected in addition to the “orthodox” antigen processing and presentation in the context of MHC class I- or II- restricted priming pathways (Donnelly *et al.* 2005; Gurunathan *et al.* 2000; Huygen 2005). Thus, how DNA vaccination can induce the two arms of the immune response has been an active area of investigation and covers three main areas: antigen presentation, the immunological properties of the plasmid backbone and the role of cytokines in inducing the immune responses (Donnelly *et al.* 2005).

1.4.1.1.1 Mechanism of antigen presentation

Processing and presentation of antigen following DNA immunization is one of the intriguing aspects of the DNA-induced immune response. The exact mechanism by which injected or particle-coated plasmid DNA leads to antigen presentation has yet to be fully defined (Porgador *et al.* 1998), but at least three mechanisms are involved, namely direct transfection of professional APCs, direct priming by somatic cells and cross-priming (Gurunathan *et al.* 2000). Current data demonstrate that bone marrow-derived APCs (e.g. dendritic cells (DC)) play a pivotal role in initiating the immune response following both IM and GG delivery of plasmid DNA (Tuting *et al.* 2000). Injection of plasmid DNA results in direct transfection of a small number of DCs that present antigen to T cells, and general activation of large numbers of DCs that were not transfected (Akbari *et al.* 1999; Casares *et al.* 1997; Porgador *et al.* 1998). The intradermal (ID) deposition of plasmid DNA may result in the activation of the APCs present in the dermis (Langerhans cells) at a level of 5% of the cellular tissue, but the situation in muscle is not clear (Condon *et al.* 1996; Porgador *et al.* 1998).

Somatic cells (myocytes, keratinocytes, or any MHC class II-negative cells) may also directly present antigens to T cells to induce primary immune responses, although they alone are not as efficient as the DCs at priming (Agadjanyan *et al.* 1999; Iwasaki *et al.* 1997a). When CTLs destroy somatic cells (mainly myocytes) expressing the antigen plasmids can be released from these myocytes and can be phagocytosed by monocytes migrating through the muscle (Davis *et al.* 1997; Egan and Israel 2002; Leitner *et al.* 1999). Thus, transfected myocytes may serve as plasmid-depots for continued APC-transfection (Corr *et al.* 1996). Additionally, with IM injections, plasmid DNA may gain access to the lymphatic or circulatory systems to transfect myocytes away from the site of injection (Torres *et al.* 1997). With GG administration, transfected DCs migrating from the epidermis to lymphoid tissues, and transfected non-migratory cells (e.g. keratinocytes and Langerhans) remaining in the epidermis, can all present antigen and influence the magnitude of the immune response (Akbari *et al.* 1999; Klinman *et al.* 1998).

The resulting proteins are not only presented to T cells by the somatic cells themselves, but also cross over into the MHC class I-restricted processing way, allowing APCs to activate both $CD8^+$ and $CD4^+$ T lymphocytes (Cohen *et al.* 1998). Such a cross-priming process, although contradicting the dogma that only endogenous antigens can enter the MHC class I pathway, may provide a principle mechanism by which plasmid DNA is delivered to cells such as myocytes and these myocytes effectively shuttle antigen to DCs or other APCs to induce CTL and humoral responses (Kumaraguru *et al.* 2000; Sharma and Khuller 2001).

The presentation by APCs of endogenously expressed antigen and exogenously acquired antigen to T cells is not independent (Tuting *et al.* 2000). Bone marrow-derived APCs are

essential for generation of the plasmid DNA-induced immune responses via ID or IM routes (Akbari *et al.* 1999; Casares *et al.* 1997; Corr *et al.* 1996; Doe *et al.* 1996; Fu *et al.* 1997b; Iwasaki *et al.* 1997b). Somatic cells may serve as a reservoir for antigen and play a secondary role in the induction of immune response via cross-priming (Gurunathan *et al.* 2000).

1.4.1.1.2 Role of CpG motifs in plasmid DNA vector backbone

Unlike inactivated vaccines, naked DNA vaccines without adjuvant can induce effective immune responses in experimental studies in animal models. Part of this effectiveness is attributable to the plasmid DNA itself, whose backbone contains unmethylated cytosine-phosphate-guanine (CpG) dinucleotides (termed CpG motifs) (Donnelly *et al.* 2005; Greenland and Letvin 2007; Sato *et al.* 1996).

Toll-like receptor (TLR) 9, present on effector cells of the immune system, can bind to and recognize such CpG motifs (Hemmi *et al.* 2000). Interaction of TLR9 with CpG motifs activates several signaling pathways and results in an an immunostimulatory cascade (Klinman *et al.* 2004), activating B-cells to proliferate or secrete antibody (Krieg 2000), eliciting innate immune response characterized by the production of interleukin (IL)-6, IL-12 or tumor necrosis factor (TNF)-α, interferon (IFN)-γ and IFN-α (Gurunathan *et al.* 2000; Klinman *et al.* 1997b), and inducing professional APCs to secrete cytokines and resultant activation of natural killer cells (NK). Thus, CpG motifs likely play an important role in the generation of a plasmid-expressed antigen specific immune response following DNA vaccination (Greenland and Letvin 2007; Klinman *et al.* 1997b).

Several lines of evidence demonstrate that CpG motifs in the plasmid backbone of DNA vaccines contribute to the immunogenicity of DNA vaccines. Vector lacking an antigen-encoding insert was still able to induce cytokine production *in vitro* (Kojima *et al.* 2002), indicating that the antigen-encoding region of the DNA vaccine was not required for the activation of an innate immune response (Klinman *et al.* 1997b). The immunogenicity of a DNA vaccine was markedly decreased by treatment with CpG inhibitors like SssI CpG methylase and was significantly increased by co-administration of exogenous CpG-containing DNA (Klinman *et al.* 1997b). Co-administering oligodeoxynucleotide (ODN) that contained CpG motifs with an antigen elicited an antibody and cellular response similar to immunization with a plasmid encoding the same antigen (Davis *et al.* 1998). Re-engineered plasmids containing additional CpG motifs in the plasmid vector backbone elicited a higher antibody response, more CTLs, and greater IFN-γ production than did the original vector (Klinman *et al.* 1997b; Krieg *et al.* 1998a; Sato *et al.* 1996). However, some studies showed that CpG motifs appeared to be limited in their ability to increase antibody and cytokine production *in vivo* and the addition of too many CpG motifs to the plasmid backbone may reduce its immunogenicity (Krieg *et al.* 1998a; Weeratna *et al.* 1998).

Although CpG-based bacterial DNA may stimulate immune responses *in vivo* and *in vitro*, coadministration of mammalian DNA can suppress such activation (Gurunathan *et al.* 2000). Some studies showed that a subset of nonstimulatory ODNs can block the immune activation induced by ODNs containing CpG motifs. An excess of non-CpG ODNs could inhibit the uptake of fluorescein-isothiocyanate (FITC)-labeled CpG ODNs and result in the inability of CpG ODNs to induce immune stimulation (Hacker *et al.* 1998). Improved

immunogenicity of a DNA vaccine could also be achieved by removing suppressive motifs (tandem repeats of GpC) from the plasmid backbone (Krieg *et al.* 1998a). These observations emphasize the complexity of the interaction between DNA sequence motifs and the immune system (Gurunathan *et al.* 2000).

Other studies have shown that DNA vaccines could induce robust immune responses in both TLR9-positive and TLR9-negative or MyD88-negative mice (Spies *et al.* 2003). Also, production of type I interferons and CXCL10 (chemokine) occurred in TLR9-deficient and MyD88-deficient mice given DNA vaccines (Okabe *et al.* 2005). These observations suggest that TLR9-receptor independent pathways can also contribute to the immunogenicity of plasmid DNA vaccines (Wagner and Bauer 2006).

In summary, a DNA vaccine vector backbone carrying CpG motifs can function as an adjuvant or immunomodulator, which may or may not have an adjuvant effect for DNA vaccines (Donnelly *et al.* 1997b). Therefore, it may be possible to tailor the type of immune response to a DNA vaccine *in vivo* towards a given pathogen or tumor by selectively engineering stimulatory motifs into the vector backbone (Gurunathan *et al.* 2000; Tuting *et al.* 2000).

1.4.1.1.3 Role of cytokines or costimulatory molecules in DNA vaccines

DNA vaccination can induce Th1- and Th-2 biased immune response, which requires induction of a number of cytokine or costimulatory molecules that play a crucial role in generation of the effector T-cell subsets and in determining the nature of the response (Gurunathan *et al.* 2000; Manoj *et al.* 2004). These cytokines and co-stimulatory molecules, either alone or in combination, have been employed to modulate the immune response generated by DNA vaccination (Manoj *et al.* 2004). They are also termed

‘biological’ adjuvants (Cohen *et al.* 1998; Kelso 1998) or ‘genetic’ adjuvants (Laddy and Weiner 2006; Scheerlinck 2001).

These immunomodulatory molecules have been divided somewhat arbitrarily into three groups: (1) cytokines - further subdivided into three groups: a) pro-inflammatory cytokines, including granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1α, IL-1β, transforming growth factor β (TGF-β), TNF-α, TNF-β, IFN-α, IL-12; b) Th1-inducing cytokines, including IFN-γ, IL-2, IL-12, IL-15, IL-18, IFN-I (αβ) (Bracci *et al.* 2006); c) Th2-inducing cytokines, including IL-4, IL-5, IL-6, IL-10, and IL-13; (2) chemokines - including monocyte chemo-attractant protein-1(MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, regulated upon activation of normal T cell exposed and secreted (RANTES)(CCL5), T cell activation protein-3(TCA), and CCL21 (Yamano *et al.* 2006); (3) co-stimulatory molecules, such as CD40, CD80, CD86 and CD154 (Manoj *et al.* 2004; Scheerlinck 2001).

DNA vaccination studies in mice showed that co-injection of plasmids encoding cytokines or co-stimulatory molecules with antigen-encoding plasmids usually resulted in increased immunoglobulin G (IgG) responses (Manoj *et al.* 2004; Scheerlinck 2001), but in some cases, the response remained unchanged or was even down regulated (Barouch *et al.* 1998).

GM-CSF has been one of the most studied genetic adjuvants (Scheerlinck 2001). By activating and recruiting professional APCs, it significantly enhanced humoral responses in mice (Xiang and Ertl 1995), in most cases GM-CSF slightly increased CTL responses. However, the GM-CSF-encoding plasmid appears to be less potent in rhesus monkeys than in mice (Kumar *et al.* 2002).

DNA vaccines co-administered with plasmids encoding IFN-γ (Chow *et al.* 1998), IL-2 (Xin *et al.* 1999), IL-12 (Kim *et al.* 1999), IL-15 (Xin *et al.* 1999) or IL-18 (Sin *et al.* 1999) showed a decreased ratio of IgG1:IgG2a, indicating that a bias towards a Th1 type immune response occurred. Moreover, co-injection of Th1-inducing cytokines induced increased CTL response to plasmid-encoded antigens (Scheerlinck 2001).

Immunization with antigen expressing plasmids mixed with plasmids encoding Th2-inducing cytokines such as IL-4 (Sin *et al.* 1999), IL-6 (Lee *et al.* 1999), IL-10 (Kim *et al.* 1998), IL-13 (Kim *et al.* 2000) tended to induce immune responses characterized by higher levels of IgG1 antibody in terms of both a higher level of total antibody and a higher ratio of IgG1:IgG2a Ab. Nevertheless, most of these cytokines (with the exception of IL-6) elicited either similar or lower CTL responses to these antigens *in vivo* (Scheerlinck 2001).

Co-delivery of a plasmid expressing CD40 ligand/trimer (CD40LT) with β-gal-encoding plasmid decreased the IgG1:IgG2a ratio (indicating a Th1 bias), enhanced β-gal-specific humoral and CTL responses and induced protective immunity to a *Leishmania major* following challenge (Gurunathan *et al.* 1998a). In addition, a number of chemokine-encoding plasmids were reported to modulate immune responses to DNA vaccines (Scheerlinck 2001).

The combination of two or more immuno-modulators has been examined to further enhance and modulate the immune response to DNA vaccines. Significant enhancement of Th1-type immune response (as measured by a lower IgG1:IgG2a ratio) was observed when a human immunodeficiency virus (HIV) DNA vaccine was co-delivered with GM-CSF-encoding plasmid and IL-12-encoding plasmid, whereas, the combination of a HIV

DNA vaccine with a plasmid-encoding GM-CSF and a plasmid-encoding IL-4 induced an increased Th2 biased response (as measured by a higher IgG1:IgG2a ratio) (Kusakabe *et al.* 2000). The influenza NP plasmid, NPo/B7-1, coexpressing the costimulatory molecule CD80 (B7-1) and a suboptimal NP antigen (NPo) had no effect on the CTL response in mice, whereas, the NP/B7-2 plasmid coexpressing NPo and the costimulatory molecule CD80 (B7-2) increased this response. The combination of either IL-12-expressing plasmid or GM-CSF-expressing plasmid with either the NPo/B7-1 plasmid or NPo/B7-2 plasmid enhanced the CTL response *in vivo*. Co-administration of GM-CSF/IL-12 plasmid expressing GM-CSF and IL-12 with the NPo/B7-1 plasmid generated a further enhancement of the CTL response whereas co-delivery with the NPo/B7-2 plasmid did not (Iwasaki *et al.* 1997a). However, co-administration of a herpes simplex virus (HSV) DNA vaccine with both CD80-encoding and CD86-encoding plasmids had a marked negative effect on the humoral response (Flo *et al.* 2000). Thus, optimal combination of costimulatory molecules and cytokines may differ for a particular plasmid immunogen depending on the type of immune response desired (Iwasaki *et al.* 1997a).

The timing between administration of the cytokine-encoding plasmid and the antigen-encoding plasmid influenced the immune response induced by a DNA vaccine (Manoj *et al.* 2004; Scheerlinck 2001).

The method by which the cytokines were co-expressed also had an impact on the plasmid-induced immune response. Although a co-expressed plasmid encoding the antigen and immunomodulator may theoretically be expressed as a fusion protein and extend the longevity of action of cytokines or co-stimulatory molecules, co-expressed plasmid vaccination did not always enhance the immune response. For instance, a

dicistronic plasmid expressing both HIV-1 gp120 and IL-2 produced a weaker gp120-specific immune response than did the HIV-1 gp120-encoding plasmid alone (Barouch *et al.* 1998). There was little evidence that co- expression of cytokine or immunomodulators with antigen gave an advantage in DNA vaccination (Barouch *et al.* 1998; Iwasaki *et al.* 1997a). In contrast, plasmids co-expressing a target gene and IL-2 or IL-4 gene appeared to induce stronger antigen-specific antibody responses than antigen-encoding plasmids alone (Inoue *et al.* 2002; Lim *et al.* 1998).

Taken together, although the effect of immunomodulators may vary depending on DNA vaccine used, delivery regimen (method, route, dose and timing of delivery) and their different combinations, it is clear that cytokines, chemokines and co-stimulatory molecules can potentially be used as a complementary strategy to improve and/or modulate DNA vaccination (Kusakabe *et al.* 2000; Manoj *et al.* 2004; Scheerlinck 2001).

1.4.1.2 Humoral immune response

Since antibodies to viral proteins were first described after IM injection of DNA vaccines encoding HA (HA-DNA) and NP (NP-DNA) of influenza virus (Ulmer *et al.* 1993), DNA vaccination has proven to be an effective means of generating humoral immune responses against various viral, bacterial, parasitic, tumor, and eukaryotic proteins (Donnelly *et al.* 1997b).

As expected, antibody responses against DNA-encoded antigens are generally dependent on the dose of DNA (Deck *et al.* 1997; Ulmer *et al.* 1994) and the number of vaccinations (Deck *et al.* 1997). However, this dependence is lost in some laboratory animal species (Galvin *et al.* 2000). In mice, marginal or undetectable antibody was induced after the

first DNA injection (Dufour 2001). However, booster immunization did increase serum antibody titres, and this was further augmented after a third immunization, but did not alter the isotypic profile of the response (Deck *et al.* 1997; Feltquate *et al.* 1997).

In some cases, such as HIV and influenza, a combination DNA vaccine comprising multiple plasmids encoding several different antigens of a pathogen may be required to induce a broader spectrum of immune responses. In this situation, it is essential that coadministration of multiple plasmids or coexpression of multiple antigens does not interfere or compete with each other resulting in the inhibition of immune responses to one of the components (Donnelly *et al.* 1997b). Although this has not been extensively investigated, a combined DNA vaccine containing seven separate plasmids was shown to be effective against influenza virus shedding in ferrets, and antibody responses against each of three different HA components were demonstrated (Donnelly *et al.* 1995a).

1.4.1.3 Cell-mediated immune response

DNA immunization via IM, ID, GG or other means has been proven to induce antigen-specific CTL responses in mice and non-human primates (Donnelly *et al.* 1997b). CTL that are capable of recognizing and killing virus-infected targets have been demonstrated against various viruses such as influenza virus (Ulmer *et al.* 1993), rabies virus (Xiang *et al.* 1995), and HIV (Liu *et al.* 1996). In studies of influenza NP in BALB/c mice, anti-NP CTL was shown to last over two years after immunization with NP-DNA (Donnelly *et al.* 1995b; Yankauckas *et al.* 1993).

Some studies showed that repeated IM immunization enhanced cell-mediated immune (CMI) response to influenza NP (Donnelly *et al.* 1995b) and HIV Env (Shiver *et al.*

1995). However, in a murine study with three GG immunizations of 4 μg each of a plasmid encoding gp120 and 1 μg each of a plasmid encoding Rev, CTL responses appeared after two immunizations, while antibody responses were detected only after a third dose, but CTL were suppressed (Fuller and Haynes 1994). The authors suggested that the suppression was related to a switch in Th cells from Th1 to Th2, as the administration of anti-IL-4 antibody prevented this loss of CTL responsiveness.

DNA vaccine studies in mice showed that generation of Th-1 like T immune response may be a general property of DNA vaccines delivered via the IM or ID route, characterised by relatively high CTL activity, the predominance of IgG2a antibodies and γ-IFN production (Donnelly *et al.* 1997b). However, DNA vaccination using GG appears to generate a Th2-like response characterized by increased IgG1 Ab and IL-4 production (Feltquate *et al.* 1997; Mor *et al.* 1995; Pertmer *et al.* 1996).

Intramuscular injection of mice with a plasmid DNA encoding NP from PR8 virus induced IgG anti-NP antibodies, a robust CTL response to the H-2K^{d}-restricted epitope 147–155, as well as protective efficacy in mice against a heterosubtypic virus challenge (Fu *et al.* 1997a; Ulmer *et al.* 1993). This protection was shown not to be mediated by antibody responses to NP (Ulmer *et al.* 1993). A further study showed that, although CD8^{+}-restricted CTLs were essential effectors in protection, NP-DNA also induced a Th1-type of CD4^{+} helper T-cell response. This was indicated by IgG 2 predominance of anti-NP antibody and with high levels of interferon-γ and IL-2, modest level of GM-CSF and little or no IL-4 or IL-10, although a lesser role of CD4^{+} cells has been reported (Ulmer *et al.* 1998). Albeit that there are conflicting results on the relative importance of CD4^{+} and CD8^{+} for protection conferred by NP-DNA, both CD8^{+} CTL and cytokine-

secreting $CD4^+$ T cells contributed to the overall protection from influenza virus challenge in mice (Epstein *et al.* 1997; Liang *et al.* 1994; Ulmer *et al.* 1998). After examination of the roles of T cell subsets in protective immunity induced by DNA vaccines encoding NP or M (M-DNA) of PR8 virus. Epstein *et al.*(2000) found that $CD8^+$ cells were not essential for the anti-viral CTL response in NP-DNA and M-DNA-immunized mice, as either $CD4^+$ or $CD8^+$ T cells alone were sufficient to promote survival and recovery after challenge.

1.4.1.4 Immunological memory with DNA vaccination

Several studies showed that DNA vaccination was capable of generating long-term antibody responses, depending on the type of antigen used in the vaccine (Deck *et al.* 1997; Gurunathan *et al.* 2000; Raz *et al.* 1994). Others studies showed that DNA vaccination can induce long-lived Th1 responses *in vivo*, as well as long-term CTL responses (Akbari *et al.* 1999; Gurunathan *et al.* 1998b).

The possible mechanisms hypothesized for sustained humoral and CMI responses after DNA vaccination are as follows: (A) persistence of antigen synthesis in transfected cells such as in follicular DCs that could serve as an antigen reservoir to maintain the immune response, though plasmid DNA may not be detected; (B) immune responses elicited by DNA immunization are antigen-independent, although plasmid DNA as well as antigen may completely disappear; (C) Memory cells generated by DNA vaccination may differ from those achieved by protein-based immunization (Akbari *et al.* 1999; Gurunathan *et al.* 2000).

1.4.1.5 Mucosal immunization

Mucosal immunity is considered an important goal to combat pathogens that enter via the mucosal route (Loehr *et al.* 2000). IM injection and GG delivery (widely used for DNA vaccination) of naked plasmid DNA induces limited secretory mucosal IgA responses (Srivastava and Liu 2003). However, formulation of DNA vaccines with cationic lipids (Mitchell *et al.* 1995; Wong *et al.* 2001), monophosphoryl A (Sasaki *et al.* 1998b), QS-21 (Sasaki *et al.* 1998c), poly(lactide-coglycolide) microparticles (Jones *et al.* 1997) or alginate (Mittal *et al.* 2000) via IM, oral, or intranasal (IN) delivery, induced marked secretory IgA response at the mucosal sites. Mucosal immunity induced by DNA vaccines was also achieved by administration via intratracheal, aerosol, or genital-tract routes (Gurunathan *et al.* 2000). Some studies demonstrated that serum IgG responses induced by mucosal immunization are comparable to those generated by systemic immunization with the same DNA constructs (Livingston *et al.* 1998; Sasaki *et al.* 1998b; Sasaki *et al.* 1998c), whereas, others showed that mucosal immunization is insufficient to induce serum IgG responses (Ban *et al.* 1997; Fynan *et al.* 1993b; Kuklin *et al.* 1997). Although the immune effect of mucosal immunization is variable, overall it appears to be superior to systemic immunization with regards to induction of mucosal IgA responses (Livingston *et al.* 1998; Sasaki *et al.* 1998b; Sasaki *et al.* 1998c). However, in some studies mucosal DNA vaccination could not prevent virus replication and persistence at the mucosa challenge site, indicating that the immunization regimen for induction of sufficient IgA response may be suboptimal or that prevention of viral replication at the mucosal site may require immune mechanisms other than secretory IgA for those viruses (Kuklin *et al.* 1998).

Plasmid DNA vaccines have been delivered by microparticles and nanoparticles as a delivery vehicle for both mucosal and systemic immunisation (Jones *et al.* 1997; Roy *et al.* 1999). Immunization via a systemic delivery route may not achieve effective mucosal immune responses (Gallichan and Rosenthal 1996; Stevceva *et al.* 2000). However, a mucosal prime-parenteral boost immunization approach demonstrated that parenteral immunization can prime for subsequent mucosal responses (McCluskie *et al.* 2002). In addition, delivery of plasmid DNA orally with attenuated enteric bacteria such as *Salmonella* or *Shigella* is being investigated as an alternative strategy for oral and other mucosal DNA immunization (Fennelly *et al.* 1999; Sizemore *et al.* 1995).

1.4.2 Advantages and limitations of DNA vaccines

1.4.2.1 Advantages

Immunization with plasmid DNA has many potential advantages over vaccination with whole inactivated virus, subunit, and recombinant vaccines as summarized in Table 1.1 (Forde 2005; Gurunathan *et al.* 2000; Laddy and Weiner 2006; Manoj *et al.* 2004; Sharma and Khuller 2001).

1. DNA vaccines are able to induce broad-spectrum immune responses based on the ability to produce antibody, CMI and mucosal immunity (Babiuk *et al.* 1999b). CMI responses elicited by DNA vaccines against conserved internal proteins of an influenza virus, together with antibodies generated to viral surface proteins, have the potential to provide cross protection against different virus strains within the same subtype (Donnelly *et al.* 1997a; Epstein *et al.* 2002; Kodihalli *et al.* 2000).

2. DNA vaccines are inherently safer than currently used protein-based vaccines. DNA vaccines are simpler to design, faster, less expensive and more consistent to manufacture. High stability and relative temperature insensitivity of plasmid DNA make it highly suitable for mass production and distribution in both industrialized and non-industrialized countries (Abdulhaqq and Weiner 2008; Dufour 2001; Forde 2005).

3. DNA immunization allows the use of sequences of interest derived directly from clinical specimens or after only limited passage in chicken embryonated eggs or mammalian cell culture (Webby *et al.* 2004). This would avoid the selection of mutants due to passages and lead to the development of vaccines to the protein antigens encoded by the exact sequence of the clinical viral isolate (Katz *et al.* 1987). In addition, endogenous protein immunogens produced by the host administered with DNA vaccines would be more similar to those found in pathogens as compared with viral proteins produced in *Escherichia coli* (*E .coli*), yeast, or insect cells. In theory, this should make DNA vaccines more antigenic and show a closer antigenic match with the associated pathogen (Donnelly *et al.* 1997b; Srivastava and Liu 2003).

4. Due to the simple structure of the DNA plasmid vector, DNA vaccines show less side effects to the host than whole virus or protein subunit vaccines. The plasmid DNA vector can be used repeatedly without inducing immune inhibition from the host (Mascola *et al.* 2005; Ulmer 2002). It has also been reported that maternal antibodies or passively transferred antibodies do not interfere with neonatal immune responses induced by DNA vaccines (Pertmer *et al.* 2000).

Table 1.1 Comparative advantages and limitations of various type of vaccines.

Factor	Sub-factor	Killed vaccine	Live attenuated vaccine	Subunit vaccine	Live vectored vaccine	DNA vaccine
Safety						
	Virus involved during production	Yes	Yes	No	Introduction of vector virus	No
	The product itself	Improperly inactivated risk	Potential to revert to pathogenicity	Safe	Individual differences	Safe
Immune response						
	Ag Presentation	MHC class II	MHC class I & II	MHC class II	MHC class I & II	MHC class I & II
	Humoral	Strong	Strong	Strong	Strong	Need improvement
	CTL	None	Strong	None	Possible	Possible
Design						
	Incoporate multi-antigen	Impossible	Impossible	Impossible	Possible	Possible
	Introduction of DIVA	Impossible	Impossible	Possible	Possible	Possible
	Cost to develop	Very expensive	Expensive	Very expensive	Expensive	Relatively inexpensive
Manufacturing						
	Techniques	Cell culture or embryonated eggs	Cell culture or embryonated eggs	Cell culture or embryonated eggs	Cell culture or embryonated eggs	Bacterial fermentation
	Production engineering	Less difficult than live virus, more difficult than DNA	Difficult	Less difficult than live virus, more difficult than DNA	Difficult	Relatively simple
	Lead time	4-9 months	4-9 months	4-9 months	4-9 months	1 month
	Quality control	More so than DNA	Requires extensive quality control	More so than DNA	Requires extensive quality control	Easy to control
Cost						
	Cost of vaccine to manufacture	Expensive	Moderate to expensive	Expensive	Moderate to expensive	Relatively inexpensive
	Storage costs	Refrigeration	Refrigeration	Refrigeration	Refrigeration	No cold chain requirements
	Need for adjuvant	Need	Need	Need	Need	Not necessary, adjuvant itself
Side effect						
		Local reaction, even lesion	Less	Local reaction, even lesion	Less	No

* Modified from (Forde 2005; Gurunathan et al. 2000; Laddy and Weiner 2006)

5. As DNA vaccines target immune responses to certain epitopes of specific antigens (Zinkernagel and Hengartner 2001), they allow the incorporation of a marker antigen in the vaccine to enable the differentiation of infected from vaccinated animals (DIVA strategy) for disease surveillance serology conducted as part of vaccination programs (Babiuk *et al.* 1999b; Dhama *et al.* 2008; Lee *et al.* 2004). Vaccine marker antigens are likely to be either exogenous by adding a marker to the vaccine or endogenous by incorporating a marker antigen gene to the same plasmid vector (James *et al.* 2008; Li *et al.* 2008).

6. The DNA plasmid is amenable to introduction of several ORFs from one or more genes, leading to potential capacity to express diverse antigens in a single plasmid vector (Abdulhaqq and Weiner 2008; Mumper *et al.* 2000; Talaat *et al.* 2001). Thus one DNA vaccine can be formulated against different serotypes of one disease or against several diseases (Talaat *et al.* 2001).

7. DNA vaccines containing unmethylated CpG oligonucleotide sequences in the plasmid backbone may have inherent adjuvant activity to improve immune responses (Abdulhaqq and Weiner 2008).

1.4.2.2 Limitations and risks of DNA vaccines

Although DNA vaccines against numerous infectious diseases in a range of animal models are well documented, only three DNA vaccines, against West Nile virus (horses), infectious haematopoietic necrosis (salmon) and melanoma (canine), have been licensed so far (Laddy and Weiner 2006; Lalor *et al.* 2008). Several factors may be responsible for their limited application to date.

One of the most significant hurdles of DNA vaccine development has been that many of the vaccines have been less immunogenic *in vivo*. For example, a macaque study showed that the level of cellular responses induced by IM injection of DNA plasmid-based vaccines to HIV-1 was found to be only one-third of that induced by adenovirus serotype 5 (Ad5) viral vector vaccines (Casimiro *et al.* 2003). Plasmid DNA-induced immune responses have proven to be much stronger in small laboratory animal models (Kodihalli *et al.* 1999; Yang *et al.* 2004) than in larger animals (Babiuk *et al.* 2003; Babiuk *et al.* 1999b), nonhuman primates (Liu *et al.* 1997) or humans (Egan and Israel 2002; Greenland and Letvin 2007; Wang *et al.* 1998).

The second major limitation of DNA vaccines given by IM administration appears to be a very high inter-individual variability in the level of foreign gene expression, which is a major problem in development of vaccines for clinical application (Mir *et al.* 1999).

Although clinical trials have shown that DNA vaccines are safe and well tolerated, a number of safety concerns have been raised about the use of DNA vaccines (Huygen 2005). These include potential integration of plasmid DNA into the host genome via either random or homologous recombination between the host and the plasmid (Hasty *et al.* 1991). However, most transfected cells are nondividing, have very limited sequence homology between plasmid and mammalian DNA (Donnelly *et al.* 1997b), and investigations in mice that received IM injections with plasmid DNA showed no evidence of integration into the host genome using very sensitive PCR tests (Nichols *et al.* 1995).

Another theoretical concern is that plasmid-mediated immune responses might result in abnormal immune responses such as autoimmune responses (Gilkeson *et al.* 1995; Steinberg *et al.* 1990), induction of immune tolerance in neonates (Gurunathan *et al.*

2000) or, via the stimulation of cytokine production, influence the host's response to other vaccines and susceptibility to infection (Donnelly *et al.* 1997b; Gurunathan *et al.* 2000; Klinman *et al.* 1997a). However, actual studies, including human volunteers exposed to plasmid DNA vaccines, indicate that the levels of autoantibodies induced by DNA vaccines have not resulted in any evidence of systematic autoimmune disease (Gurunathan *et al.* 2000; Mor *et al.* 1997). Also, to date, IM administration of DNA vaccines in multiple studies has not shown evidence of generation of tolerance in young adult animals (Donnelly *et al.* 1997b) . Despite increasing use of DNA vaccines and cytokine-encoding plasmids, there have been no serious side effects indicative of immune system disruption reported (Ishii *et al.* 1999a; Ishii *et al.* 1999b).

1.4.3 Approaches to enhance or optimize DNA vaccines

To overcome the limitations with respect to immunogenicity of DNA vaccines, various strategies have been used to improve the level and reliability of gene expression. These have targeted various points from *in vivo* delivery into cells, through to enhancing expression of the antigen from the cell and enhancing immune responses by use of adjuvants, as discussed below.

1.4.3.1 Optimization of the plasmid vector

The DNA vaccine vectors used for expression of the antigen can have a huge impact on immunogenicity (Miller *et al.* 2004). It is generally believed that the expression level of antigen-encoding genes *in vivo* following DNA vaccination correlates positively with the plasmid DNA-induced immune response (Donnelly *et al.* 2003; Greenland and Letvin 2007; Montgomery *et al.* 1994).

1.4.3.1.1 Promoters

A promotor facilitates the transcription of a particular gene (Laddy and Weiner 2006). Promoter strength remains a critical factor to be considered in plasmid vaccine design (Miller *et al.* 2004). Viral promoters with broad cell type specificity, such as the cytomegalovirus immediate early gene (CMV/IE) promoter, simian virus 40 (SV40), murine leukemia virus (SL33) and Rous sarcoma virus (RSV) promoters, have been the most frequently used vehicles for driving the expression of the vector-encoded gene (Hasan *et al.* 1999; Manoj *et al.* 2004). In many systems the CMV/IE promoter has provided the strongest gene expression *in vivo* among the above promoters (Lee *et al.* 1997). Tissue-specific promoters used in the construction of DNA vectors, such as the muscle-creatine kinase (MCK) promoter (Bojak *et al.* 2002; Gebhard *et al.* 2000), and muscle-specific desmin (Loirat *et al.* 1999), are expected to be safer than viral promoters, but they induced lower levels of antigenic protein expression and weaker immune responses (Nettelbeck *et al.* 2000). Considering the advantages of both viral and tissue-specific promoters, some new non-viral, synthetic, and chimeric promoters have started to be investigated (Garg *et al.* 2004; Tornoe *et al.* 2002; Vanniasinkam *et al.* 2006).

1.4.3.1.2 Modified plasmid vectors

Selection of an appropriate vector is one of the most important issues in optimizing a DNA vaccine. The DNA vaccine vector is central to high level gene expression and subsequent immune response to its encoded antigen (Abdulhaqq and Weiner 2008). Some vectors are commercially available, but others with improved properties are being developed. Examples of current DNA vaccine vectors are described below.

1.4.3.1.2.1 Bidirectional and biocistronic plasmids

DNA vaccines have the potential to express multiple antigens from one or more pathogens in a single vector (Donnelly *et al.* 1997b). Two kinds of plasmids have been constructed. Bidirectional plasmids allowed coexpression of two antigens *in vitro*, which was in accord with increased immune response *in vivo* (Kwissa *et al.* 2000). The disadvantage of this plasmid was competition for gene expression from the promoters, plasmid instabilty due to the presence of more than one expression cassette and possible lower transfection efficacy of such plasmids. Bicistronic plasmids can transcribe from a single promoter and express proteins from a single mRNA (Barouch *et al.* 1998; Manoj *et al.* 2003a). However, the disadvantage was variable translational efficiency of the internal ribosome entry site (IRES), which may result in reduced expression and weaker immune responses (Mizuguchi *et al.* 2000).

1.4.3.1.2.2 Antibiotic free plasmids

Antibiotic resistance genes are primarily inserted into the plasmid for selection purposes during cloning. Owing to concern regarding the potential for generating microbial resistance, the incorporation of antibiotic resistance genes into DNA vectors for vaccination purposes is not favoured by vaccine producers or vaccine regulatory agencies. Thus novel plasmids without antibiotic resistance genes based on "operator-repressor titration" are currently being investigated (Cranenburgh *et al.* 2001; Williams *et al.* 1998).

1.4.3.1.2.3 Simple MIDGE vectors

The minimalistic immunogenically defined gene expression (MIDGE) vector is another example of an antibiotic resistance gene-free plasmid. These vectors contain the minimal gene expression elements including a promoter/intron, gene of interest and

polyadenylation signal. Owing to the elimination of bacterial DNA sequences, unfavorable side effects (potential integration into host, antibiotic resistance) of plasmid DNA are excluded (Manoj *et al.* 2004; Schakowski *et al.* 2001).

MIDGE vectors expressing cytokines together with cytotoxic T lymphocyte antigen-4 (CTLA-4) have been utilized as adjuvants for vaccination studies (Muller *et al.* 2002). Their potential to replace plasmid vectors is dependent on their transfection efficiency. Linking nuclear localization signal (NLS) peptides with the MIDGE vector resulted in enhanced antibody responses using IM delivery (Schirmbeck *et al.* 2001). Thus, MIDGE vectors appear to be attractive candidates for DNA vaccines to efficiently prime both humoral and cellular antiviral immunity.

1.4.3.1.3 Codon bias

The genetic code is almost universal to all organisms, but coding sequences in DNA in different species do not use synonymous codons with equal frequencies. This is dependent on which tRNA pools are rate limiting in a particular species (Grosjean and Fiers 1982; Laddy and Weiner 2006). Generally speaking, there is a high correlation between codon usage, tRNA abundance and the level of gene expression (Duret 2000). The genetic composition of the gene appears to impact on the expression of genes in a heterologous system, which is attributable to the existing tRNA pool that is available for translation of a paticular gene in eukaryotic cells (Saier 1995). Many pathogens have a very different codon bias and/or genomic GC content as compared with mammals (Doria-Rose and Haigwood 2003; Ikemura 1985). In the context of DNA vaccines, this bias may result in inefficient translation, low expression levels of microbial genes in transfected mammalian cells, and hence low immunogenicity of DNA vaccines (Garmory *et al.* 2003;

Manoj *et al.* 2004). Thus, optimizing codons in plasmid-encoded genes may become an approach for enhancing efficacy in genetic immunization. A number of studies have demonstrated enhanced antibody and CTL responses through the codon optimization of the antigen-encoding DNA (Manoj *et al.* 2004). However, re-engineering the coding sequence of a model protein (Thy-1) using ubiquitous HIV codons significantly impaired Thy-1 expression, whereas, altering the coding sequence of the jellyfish green fluorescent protein (GFP) gene to reflect codon usage in the human genome resulted in increased expression in mammalian cells (Haas *et al.* 1996). Also using HIV codon optimization resulted in the removal of inhibitory sequences, which reduce the export of RNA from the nucleus as well as translation levels (Haas *et al.* 1996).

An additional mechanism resulting from codon usage optimization may contribute to increased immunogenicity. Altering the coding sequence of particular genes to conform to the preferred mammalian codons resulted in the introduction of multiple CpG motifs into the plamid backbone (Doria-Rose and Haigwood 2003). As unmethylated CpG is known to be immunostimulatory, CpG-rich plasmids without insertion of foreign gene can be used as adjuvants (Klinman *et al.* 1997b; Sato *et al.* 1996).Thus, a codon-optimized gene may have an inherent adjuvant effect due to increased CpG motifs, though this is not always the case (Krieg *et al.* 1998b).

Apart from codon usage bias, other important factors may also influence gene expression at the RNA molecule level (Laddy and Weiner 2006). Sequences rich in G+C content probably form secondary structures which can hinder protein translation. Internal splice sites can result in the production of incomplete or unwanted antigens. Cis-acting elements can inhibit protein synthesis, such as the rev-responsive element (RRE) in the HIV

protein Gag. Removal of RRE resulted in high-level protein expression (Indraccolo *et al.* 1998; Muthumani *et al.* 2003). In addition, modifications of native leader sequences, which affect mRNA translation and protein secretion, may have marked effects on translation efficiency (Kutzler *et al.* 2005).

1.4.3.1.4 Kozak sequences

One control point that can influence protein synthesis from plasmid vectors is at the stage of translation of mRNA transcripts (Babiuk *et al.* 2003). A comparison of several hundred mRNA sequences showed that presence of the CCA/GCCAUGG consensus sequence, named Kozak sequence, around the initiator codon was important for efficient initiation of translation in higher eukaryotes (Kozak 1987b). A suboptimal sequence flanking the AUG initiation codon influences its recognition by eukaryotic ribosomes and leads to “leaky scanning” during the translation process (Kozak 2005). Prokaryotic genes and some eukaryotic genes do not contain Kozak sequences, therefore, the incorporation of a Kozak sequence into a plasmid vector backbone may increase the expression level of the transgenes in the context of DNA vaccines (Garmory *et al.* 2003).

1.4.3.1.5 Other elements

Intron and polyadenylation signals can also affect the level of expression of the antigen. The addition of an intron, such as the intron A of the CMV/IE gene (Chapman *et al.* 1991), and polyadenylation signals, such as bovine growth hormone (Braun *et al.* 1998), β-globin IVS (Collings *et al.* 1999) and SV40 minor t antigen (Herrera *et al.* 2000), to the plasmid led to increased protein expression. Due to the presence of two potential CpG islands in the ampicillin resistance (Amp^r) gene and absence of CpG islands in the

kanamycin resistance (Kan^r) gene, Kan^r-expressing plasmids appeared to induce a lower immunostimulatory effect than Amp^r-expression plasmids (Sato *et al.* 1996).

1.4.3.2 Immunization regimen

1.4.3.2.1 Delivery methods

Numerous studies have shown that the method of DNA immunization can affect both the strength and the nature of immune responses (Deml *et al.* 2001). For the purpose of this review and this study, delivery methods refer to the use of devices to drive plasmid DNA into the host. It can be classified into two categories: syringe and needle, and needle-free devices, such as biojecto (Jackson *et al.* 2001; Williams *et al.* 2000), powderject (Chen *et al.* 2000a), GG (Fynan *et al.* 1993b; Pertmer *et al.* 1996), electroporation (EP) (Hirao *et al.* 2008; Selby *et al.* 2000), and topical application devices (Liu *et al.* 2001; Watabe *et al.* 2001). Needle injection is easily and rapidly performed and the DNA is prepared for injection simply by resuspension in a saline solution (Barry and Johnston 1997; De Rose *et al.* 2002; Webster *et al.* 1994). Needle-free devices for DNA immunization have been more efficient in generating immune responses. Studies in mice showed that the amounts of DNA required for a comparable antibody response via GG delivery were approximately 100-fold less than that for needle injection (Babiuk *et al.* 2003; Barry and Johnston 1997; Doria-Rose and Haigwood 2003; Pertmer *et al.* 1995).

Although biojector and powderject have been used to deliver live and subunit vaccines as well as DNA vaccines, improvements in the transfection rate of host cells with plasmid DNA are still required (Chen *et al.* 2000a; Degano *et al.* 1998; Rogers *et al.* 2001). Use of GG and EP has been shown to give efficient uptake of plasmid after immunization (Tang *et al.* 1992; Zucchelli *et al.* 2000). GG-mediated delivery has been extensively

used in DNA vaccination in a number of animal models, but it appears to produce a Th-2 type immune response, which may not protect the host from infections in all instances (Jankovic *et al.* 2001). Furthermore, owing to limitations in the amounts of plasmid DNA that can be coated onto the gold particles, multiple shots by GG are usually required (Barry and Johnston 1997; Manoj *et al.* 2004).

EP can be combined with commonly used methods of vaccine delivery. Intramuscular or ID injections followed by EP resulted in an increased immune response in mice and pigs (Drabick *et al.* 2001; Glasspool-Malone *et al.* 2000; Mathiesen 1999; Selby *et al.* 2000; Widera *et al.* 2000). Intradermal injection of naked DNA with EP in the presence of nuclease inhibitors synergistically enhanced transgene expression and subsequent immune response (Drabick *et al.* 2001; Glasspool-Malone *et al.* 2000; Glasspool-Malone *et al.* 2002).

Nevertheless, the disadvantages of device-dependent delivery methods may restrict or limit their future application. These include the limited amount of DNA vaccine administered, the requirement for multiple shots, the effect of the noise accompanying a GG shot, the requirement for a helium supply as the propellant for the GG, the need for an apparatus for generation of electronic pulses, tissue necrosis potentially caused by EP and the cost-effectiveness of these methods (Glasspool-Malone and Malone 2002; Manoj *et al.* 2004). The topical application without needle injection has a clinical advantage, but complicated operating procedures involved also limit its application (Fan *et al.* 1999; Watabe *et al.* 2001).

1.4.3.2.2 Delivery routes

A range of routes has been examined for the administration of DNA vaccines. Delivery routes can be broadly grouped into two categories: mucosal and systemic. The former routes include IN (Fynan *et al.* 1993b), ocular/orbital (McCluskie *et al.* 1999), eyedrop (Russell and Mackie 2001), oral (McCluskie *et al.* 1999), sublingual (SL) (McCluskie *et al.* 1999), intrarectal (McCluskie *et al.* 1999), intravaginal (IVG) (McCluskie *et al.* 1999), intraperineal (IPER)/intravulvomucosal (McCluskie *et al.* 1999), intratracheal (Glasspool-Malone *et al.* 2002), intrajejunal (Etchart *et al.* 1997), intrabursal (Fynan *et al.* 1993b), and via Peyer's patches (Schubbert *et al.* 1997). Systemic routes include IM (McCluskie *et al.* 1999), ID (Fynan *et al.* 1993b; McCluskie *et al.* 1999), intravenous (IV) (Fynan *et al.* 1993b; McCluskie *et al.* 1999), intraperitoneal (IP) (Fynan *et al.* 1993b; McCluskie *et al.* 1999), intralymphatic (Maloy *et al.* 2001), and subcutaneous (SC) (Fynan *et al.* 1993b).

The magnitude of DNA vaccine-induced immune responses has been shown to be dependent on the delivery route. The delivery routes of plasmid DNA have also been shown to influence the type of immune response (Manoj *et al.* 2004). In many cases, IM injections of plasmid DNA by needle elicit a Th1-type response, whereas,, ID administration by needle injection generates a mixed immune response. By contrast, GG delivery appears to predominantly yield a Th2 response (Feltquate *et al.* 1997; Leitner *et al.* 1997; Leitner *et al.* 1999; Manoj *et al.* 2004).

Lymph nodes have also been explored as sites of DNA vaccine delivery. Mice given a plasmid expressing a CTL epitope of lymphocytic choriomeningitis virus (LCMV) via the intralymphatic and intrasplenic routes elicited a $CD8^+$cytotoxic T lymphocyte

response, which was 100- to 1,000-fold more efficient than that induced by ID or IM immunization (Maloy *et al.* 2001).

Additionally, tattoo immunization (a new rapid and potent ID DNA vaccination) of murine skin with plasmid DNA has been shown to provide a highly transient pulse of vaccine antigen that induced strong protective immune responses within a relatively short time period (Bins *et al.* 2005).

1.4.3.2.3 Delivery dose, number and timing of doses, and volume

The delivery dose, number, volume and timing of doses of plasmid DNA may affect the immune response resulting from DNA vaccination.

The delivery dose required depends on the delivery method and route used as well as the nature of the plasmid vector and its ability to express the antigen in the target cells. Typically, in small laboratory rodents, immunization via IM or SC route requires 10 to 100 μg of plasmid DNA to induce immune responses, whereas, 0.1-1 μg of plasmid DNA is required to induce antibody or CTL responses by GG (Gurunathan *et al.* 2000). In large animals, primates or humans, higher doses of DNA appear to be required (Doria-Rose and Haigwood 2003; Manoj *et al.* 2004). However, studies in cattle (Cox *et al.* 1993) and chimpanzees (Davis *et al.* 1996) showed that, irrespective of the DNA delivery route, it may not be necessary to increase the dose proportionate to the body size for larger animals (Davis 1997; Leitner *et al.* 1999). One study with a HIV DNA vaccine in macaques showed that increasing the vaccine DNA dose did not generate the expected higher immune responses to HIV-1 and other factors such as the use of a strong promoter or co-administration of cytokins or other stimulating molecules may be required for higher protein expression from DNA vaccines (Galvin *et al.* 2000).

The number of doses also influences the immune response for DNA vaccines. For a very immunogenic gene such as the rabies glycoprotein (Lodmell *et al.* 2001) and influenza HA (Robinson *et al.* 1997), a single dose of DNA vaccine may induce antibody and CTL responses, whereas, for less immunogenic genes, it may be difficult to induce protective immune responses from a single dose (Doria-Rose and Haigwood 2003). Nevertheless, in most cases, the immune responses can be enhanced by successive boosting with the vaccine (Gurunathan *et al.* 2000). For instance, neutralizing antibody to HIV is usually detected only after multiple doses of DNA vaccine (Barnett *et al.* 2001). Besides an increase in the magnitude of the immune response multiple immunizations with DNA vaccines may alter the type of response induced (Fuller and Haynes 1994). In a macaque study using DNA vaccination against simian immunodeficiency virus (SIV), antibodies to SIV were reduced after the sixth and seventh vaccinations but were accompanied by increased CTL responses (Doria-Rose and Haigwood 2003).

The time intervals between DNA immunizations may also have an effect on plasmid-induced immune responses. In a study using the Powderject GG to deliver SIV vaccine to macaques, increasing the length of the resting period between immunizations and reducing the number of immunizations achieved substantially better responses (Fuller *et al.* 1997).

Distribution of DNA vaccines significantly influenced their immunogenicity following IM injection in mice (Davis *et al.* 1993b; Dupuis *et al.* 2000). Larger injection volumes in mice appear to result in higher hydrostatic pressure, causing a higher uptake of plasmid DNA and generating stronger immune responses (Dupuis *et al.* 2000). Proportionally large volumes simply cannot be administered in larger animals (Greenland and Letvin

2007; Liu *et al.* 1999). Correspondingly, higher dose rates of plasmid DNA in large animals appear to be needed to generate immune responses than that in small laboratory animals (Doria-Rose and Haigwood 2003).

Because the immunogenicity of DNA vaccines may be limited by delivery-related problems, it is important to optimize the immunization regimens for the target antigen and host species. This also requires consideration of the practicality of the method including the route, number and volume of doses and their timing.

1.4.3.3 Co-administration of adjuvants with DNA vaccines

Naked plasmid DNA immunogens have the potential to elicit potent cellular and humoral immune responses against infectious agents. Although plasmid DNA itself can stimulate non-specific production of cytokines that augment specific immune responses (Sato *et al.* 1996; Srivastava and Liu 2003), DNA-induced immune responses can be further enhanced or modulated by the use of immuno-stimulatory adjuvants or improved delivery formulations (Greenland and Letvin 2007). Adjuvants for plasmid DNA vaccines can broadly be divided into two classes, genetic and chemical adjuvants based on their origin (Sasaki *et al.* 2003). Genetic adjuvants are vectors expressing cytokines or other co-stimulatory molecules, and are species-specific. As veterinary vaccines work across different species and only minimal knowledge of immune modulators such as cytokines and interleukins for many species is available, adjuvants dependent on universal activation signals of the innate immune response are to be preferred (Scheerlinck and Greenwood 2006). Chemical adjuvants can be expected to function similarly in different

species and at this time show greater promise for animal DNA vaccines than genetic adjuvants.

1.4.3.3.1 Genetic adjuvants

A number of studies have demonstrated that the co-delivery of plasmid DNAs expressing immunomodulatory molecules as mentioned in section 1.4.1.1.3, along with DNA vaccines can enhance the magnitude and the nature of the immune response to DNA vaccines in experimental animal models (Egan and Israel 2002).

1.4.3.3.2 Chemical adjuvants

Although naked DNA vaccines themselves can induce immune responses, biodistribution studies showed that after IM injection only a small fraction of the injected DNA transfected target cells which resulted in poor immunogenicity, especially in larger animals and humans (Donnelly *et al.* 2005).

Chemical adjuvants can function as activators of innate immunity (Kovacsovics-Bankowski *et al.* 1993; Leifert *et al.* 2004), provide slow release depots, or alter immune cell trafficking (Sigal *et al.* 1999). They can generally be sorted into several categories (Table1.2), most of which have shown promise for enhancing the expression and immunogenicity of plasmid DNA vaccines in animal models (Greenland and Letvin 2007).

Agents that cause muscle necrosis, such as cardiotoxin or bupivicaine, increase immune responses to DNA vaccines through increased protein expression in regenerating myocytes and recruitment of APCs by inflammatory responses (Coney *et al.* 1994; Davis *et al.* 1993a).

Table 1.2 Applications of chemical adjuvants in DNA vaccines.

Adjuvant type	Examples	Vaccine and reference
Conventional adjuvants		
Mineral salts	Aluminum phosphate, Aluminum hydorxide	V1JpHA encoding HA from influenza virus A/Georgia/03/93; V1Jns/gD and V1Jns/DgB encoding *herpes simplex virus* 2 (HSV-2) gD and the amino-terminal 707 amino acids of gB (Ulmer *et al.* 1999)
	Calcium phosphate	Plasmid encoding an H5 AIV HA gene (Suarez and Schultz-Cherry 2000a)
Tensoactive compounds	QS-21	Plasmid encoding HIV-1 *env* and *rev* gene (Sasaki *et al.* 1998c)
Microorganism-derived adjuvants	Monophosphoryl lipid A(MPL)	DNA vaccine encoding the CVS rabies virus G (Lodmell *et al.* 2000)
	Cholera toxin	Plasmid pPJV7418 encoding for hepatitis B surface and core antigens, Plasmid pC-Env/T encoding a truncated HIV-1 gp120 gene, pM2-FL encoding the M2 gene of influenza A/Sydney/5/97 (H3N2) virus (Arrington *et al.* 2002)
Others	Cardiotoxin	Plasmid encoding hepatitis B surface antigen (HBsAg) (Davis *et al.* 1993a)
	Bupivicaine	Plasmid encoding HIV-1gp160 (Coney *et al.* 1994)
	Barium chloride	β-galactosidase (β-gal)-encoding plasmid (Wells *et al.* 1998)
	Dimethyldioctadecylammonium bromide (DDA)	Plasmids encoding pseudorabies virus glycoprotein B (gB) and gD (van Rooij *et al.* 2002)
	Sucrose	Plasmid encoding an H5 AIV HA gene (Suarez and Schultz-Cherry 2000a)
Particulate delivery system		
Liposomes/ Lipid particles	Cationic liposomes	Cystic fibrosis transmembrane conductance regulator-encoding plasmid (Hyde *et al.* 2000), Plasmid DNA encoding HBsAg (Perrie *et al.* 2001)
	Anionic liposomes	Tyrosinase-related protein-2 (TRP2)-encoding plasmid (Yamano *et al.* 2006)
	DermaVir	Simian-human immunodeficiency virus (SHIV) plasmid pSHIV(int-) (Lisziewicz *et al.* 2005)
Polymers	Polyethyleneimine (PEI)	HIV-glycoprotein 120 (gp120)-encoding plasmid (Garzon *et al.* 2005)
	Dimethylaminoethyl methacrylates(DEAEMA)	DNA plasmid (pCMV-S) encoding HbsAg (Bos *et al.* 2004)
	β-Cyclodextrin	Plasmids pGL3-CVencoding for the luciferase gene and pEGFPLuc coding for an EGFP/luciferase fusion protein (Pun *et al.* 2004)
	Cationic poly(β-amino esters)	pVRC-HIV-1 Env IIIB gp120 (Greenland *et al.* 2005)
	Poloxamers	HIV-1 gag-encoding plasmid (Evans *et al.* 2004)
	Polyvinylpyrrolidone (PVP)	Human growth hormone or bacterial β-gal)-encoding plasmids (Anwer *et al.* 1999)

Table 1.2 Applications of chemical adjuvants in DNA vaccines.(continued).

Adjuvant type	Examples	Vaccine and reference
Nano- and microparticles	Poly(dl-lactic-*co*-glycolic acid) (PLGA)	Plasmid encoding multiple HIV-1 CTL epitopes and several Simian immunodeficiency virus (SIV) epitopes together with the SV5 Pk tag epitope (Sharpe *et al.* 2003)
	PLGA-CTAB	Plasmid encoding HIV p55 gag (Denis-Mize *et al.* 2003)
	PLGA-poly(β-amino ester)	Plasmid DNA encoding firefly luciferase, β-gal, or SIY/β-gal fusion (Little *et al.* 2004)
	Alginate	Plasmid encoding bacterial β-gal (LacZ) gene (Mittal *et al.* 2000)
	Chitosan	Plasmid encoding the CTL epitope from respiratory syncytial virus M2 protein (Iqbal *et al.* 2003)
	Poly(*ortho* ester) polymers	pCMV-Luc expressing luciferase and pCI-Neo expressing the SIY antigen (Wang *et al.* 2004a)
Synthetic adjuvants	Synthetic peptide Trp-Lys-Tyr-Met-Val-d-Met(WKYMVm)	pGX10-GE encoding HIV-1 tat gene, pGX10-NP encoding the NP gene of PR8 virus, pGX10-HA encoding the HA gene of influenza A/WSN/33 virus, and pGX10-gDsS encoding the S gene of hepatitis B virus (HBV) (Lee *et al.* 2005)
	Ubenimex	Plasmid encoding HIV-1 (Sasaki *et al.* 1998a)
	Oligodeoxynucleotides (ODNs) containing CpG motifs	Plasmid encoding infectious bursal disease virus (Wang *et al.* 2003)

Aluminum salts are the most widely used adjuvant for both human and veterinary vaccines (Lindblad 2004). Aluminum phosphate possibly acts by recruiting APCs to the site of the IM injection, where a fraction of transfected muscle cells expressed a plasmid-encoded antigen (Donnelly *et al.* 2005). Formulation of plasmid DNA vaccines with aluminum salts has been shown to enhance humoral immune responses in rodents and primates (Ulmer *et al.* 1999).

Liposomes are synthetic spheres possessing lipid layers that can encapsulate antigens and act as both a vaccine delivery vehicle and adjuvant (Petrovsky and Aguilar 2004). These delivery vehicles not only protect plasmid DNA from nuclease degradation, but also facilitate the transfer of DNA across membranes and release DNA contents following

fusion with endosomes (Nakanishi and Noguchi 2001). Either cationic or anionic liposome formulations, alone or in combination, can augment immune responses to plasmid DNA immunogens (Perrie *et al.* 2001). Furthermore, the immunogenicity of liposome formulated plasmid DNA can be augmented in combination with cytokine adjuvants (Yamano *et al.* 2006).

A variety of cationic polymers (Greenland and Letvin 2007) have shown promise for enhancing the expression of plasmid DNA by facilitating transport of plasmid DNA across cell membranes (Ledley 1996), reducing DNA degradation and facilitating its release from endosomal compartments (Lungwitz *et al.* 2005). The polymer may also act in part through a local inflammatory response (Donnelly *et al.* 2005). Nevertheless, formulation with cationic polymer polyethyleneimine (PEI) decreased the immune responses generated by intramuscularly administered plasmid DNA encoding diphtheria toxin (Anderson *et al.* 2004).

Microparticle complexes appear to improve delivery of DNA to APCs by facilitating trafficking to local lymphoid tissue and facilitating uptake by DCs (Denis-Mize *et al.* 2003; Dupuis *et al.* 2000). In particular, the copolymer poly-DL-lactic-*co*-glycolic acid (PLGA) has been extensively investigated as a carrier for plasmid DNA immunogens (Cui and Mumper 2003b). Poly-lactide-*co*-glycolide (PLG) delivery of HIV DNA vaccines was shown to be effective at inducing antibody and CMI responses in various species, including rhesus macaques (O'Hagan *et al.* 2004). Recently, nanoparticles have shown promise for enhancing immune responses to plasmid DNA vaccines (Cui and Mumper 2002a, b, c, 2003a; Li *et al.* 2009; Lori *et al.* 2007).

Conventional adjuvants effective for inactivated whole organism and subunit vaccines may sometimes enhance the immune responses induced by DNA vaccines in large animal species. However, most studies in the mouse model have shown that formulation of DNA vaccines with such adjuvants is usually not beneficial (van Drunen Littel-van den Hurk *et al.* 2004). Hence, there is a need to evaluate new adjuvant formulations and delivery systems for DNA vaccines (Greenland and Letvin 2007; van Drunen Littel-van den Hurk *et al.* 2004; Wales *et al.* 2005).

1.4.3.4 Antigen targeting to improve DNA vaccines

1.4.3.4.1 Extracellular targeting

As APCs play a crucial role in the generation of immune responses, connecting the plasmid-encoded antigens with molecules that recognize receptors on APCs is one approach to effectively target limited antigens to give effective antigen presentation. Molecules to which plasmid-encoded antigens have been linked have included anti-CD11c, anti-CD40, anti-HLA-DR, anti-MHC II, CD154, cytotoxic T-lymphocyte antigen (CTLA)-4, IgG-Fc, L-selectin, p53TCR-PE38 and Troy Bodies (Manoj *et al.* 2004; Scheerlinck 2001). Another molecule used for targeting is C3d, which recognizes its receptor CR2 (complement type 2 receptor) or CD21 on B cells. Although this induced stronger immune response in some cases (Manoj *et al.* 2003b; Ross *et al.* 2001; Smahel *et al.* 2001) 146, 151), this strategy is not always successful (Frleta *et al.* 2001; Suradhat *et al.* 2001). Interestingly, DNA vaccination using antigen targeting to APCs did not show direct correlation between increased antibody level and higher protective immune response for *Corynebacterium pseudotuberculosis* in sheep (Chaplin *et al.* 1999).

1.4.3.4.2 Intracellular targeting

Ubiquitin has been linked with the antigen-encoding sequences to facilitate plasmid-encoded antigens being targeted to proteosomes, where antigen is rapidly degraded and presented via the MHC class I pathway (Rodriguez *et al.* 2001). Alternatively, linking the antigen to viral protein 22 (VP22) from viruses, such as HSV-1, bovine herpesvirus 1 (BHV-1) and Marek's disease virus type 1, effectively transported proteins synthesized in one cell to neighbouring cells, which was expected to increase the number of cells presenting the antigen (Manoj *et al.* 2004). These approaches have been shown to enhance CTL responses (Elliott and O'Hare 1997; Fu *et al.* 1998; Hung *et al.* 2001; Hung *et al.* 2002; Leachman *et al.* 2002; Oliveira *et al.* 2001), but are not always effective (Vidalin *et al.* 1999).

Linking specific protein epitopes with the lysosome integral membrane protein (LIMP-II) was used to target lysosomal compartments for the induction of immune responses via the MHC class II pathway (Rodriguez *et al.* 2001). Short peptide sequences linking to the protein transduction domain (PTD) resulted in rapid trafficking to the surfaces of transfected cells. The plasmid encoding the PTD-linked epitope induced a markedly accelerated $CD8^+$ T cell response (Leifert *et al.* 2001).

1.4.3.5 Prime-boost strategies

A number of studies have demonstrated that in many cases, a combination of two or more vaccine modalities generate better immune responses than either vaccine alone (Doria-Rose *et al.* 2003; Klinman *et al.* 1998). Priming with DNA and boosting with other vaccines, or vice versa, enhanced either Th1- (Hanke *et al.* 1999; Li *et al.* 1998) or Th2-

type (Barnett *et al.* 1997) biased immune responses. This approach can also avoid one major drawback for recombinant virus-based vaccines where booster vaccination with these vaccines may be ineffective due to an anamnestic responses to the vector itself (Restifo and Rosenberg 1999). DNA vaccines coupled with other types of vaccines have conferred some protection against a diverse range of pathogens in animal models as discussed below (Doria-Rose and Haigwood 2003). For HIV and malaria, prime-boost vaccination regimens have successfully progressed to clinical trials in humans (Giri *et al.* 2004; Moore and Hill 2004).

One combination involves initial use of DNA vaccine followed by a subsequent vaccination of exogenous protein with adjuvant and this gives better immunogenicity than repeating the DNA vaccination (Tanghe *et al.* 2001; Wang *et al.* 2004c). However, cattle primed with MPB70 DNA and boosted with MPB70 protein generated a strong antibody response and a weak IFN-γ response, but were not protected against bovine tuberculosis (Wedlock *et al.* 2003).

Vaccination regimes using initial DNA vaccination followed by secondary vaccination with recombinant pox-virus (Robinson *et al.* 1999; Sedegah *et al.* 2003), vaccinia virus (McConkey *et al.* 2003; McShane 2002; Sedegah *et al.* 1998), adenovirus (Lo *et al.* 2008; Wang *et al.* 2004b), live *Salmonella typhimurium* vaccine (Mollenkopf *et al.* 2001) or live attenuated influenza virus (Huber *et al.* 2009) have all conferred potent and promising immune protection.

Regimes where DNA vaccinations have been followed by secondary vaccination with inactivated virus particles have not been as effective in protecting macaques against SHIV challenge as vaccinia virus priming/DNA boosting or DNA priming/ vaccinia virus

boosting (Doria-Rose *et al.* 2003). Heterologous HA-DNA vaccine priming with inactivated trivalent influenza vaccine (TIV) boost was more effective in eliciting antibody responses against H1 or H3 serotype influenza viruses in rabbits than homologous prime/boost (DNA/DNA or TIV/TIV) (Wang *et al.* 2008a).

Mice mucosally primed with recombinant viral vector and boosted with DNA vaccine, both encoding the gB protein of HSV, elicited better mucosal and systemic responses (Eo *et al.* 2001). Recombinant vaccinia virus priming and DNA boosting provided significant protection from SHIV mucosal challenge in macaque models (Doria-Rose *et al.* 2003).

1.4.3.6 CpG motif-based adjuvants

Three main approaches have been taken to use the adjuvant effects of CpG motifs in DNA vaccines and these are currently being investigated (Aguilar and Rodriguez 2007). They include immunostimulatory ODNs (co-administered *in vivo*) (Marshall *et al.* 2005), ODN antigen conjugates (an ODN chemically conjugated to an antigen) (Datta *et al.* 2004) and DNA vaccine vectors with enhanced immunostimulatory sequences (ISS) sequences (engineering the CpG motifs into the plasmid DNA backbone) (Rodriguez *et al.* 2003). A number of studies showed that plasmid DNAs in combination with CpG-containing sequences enhanced immune responses to plasmid immunogens, although co-delivery of CpG-containing ODNs with DNA vaccines appeared to have a negative effect (Weeratna *et al.* 1998).

1.4.4 Second-generation DNA vaccines

In addition to consideration of plasmid vectors, immunization regime and adjuvant systems to enhance immune responses and protection from DNA vaccines, consideration

has been given recently to optimizing the part of the target gene to be expressed and also to the use of some novel delivery systems with the aim of getting more consistent and robust immune responses. This is leading to the development of second-generation DNA vaccines (as compared to classic full-length gene-expressed plasmid DNA) and some examples are described below.

1.4.4.1 Truncated DNA vaccines

In some cases, full-length genes have not been found to be suitable as DNA vaccine candidates because they either express highly immunogenic protein sequences that are not specific or protective (Chen *et al.* 1998a), are toxic for the host (Barry and Johnston 1997) or are immunosuppressive in the host (Levy 1993). However, use of a DNA vector containing a truncated gene has given enhanced immune response. An example is a plasmid vector vaccine containing the soluble or membrane-bound truncated forms of the neu oncogene, after the removal of the cytoplasmic kinase domain from the full-length gene, which generated better protective antitumor immunity than the vector encoding the full-length gene (Chen *et al.* 1998a).

1.4.4.2 Epitope-based vaccines

Multi-epitope DNA vaccines, sometimes referred to as polyepitope or polytope vaccines, have been constructed by linking well-defined epitope areas of genes together with appropriate nucleotide spacers (An *et al.* 2000; Leifert *et al.* 2004; Suhrbier 1997; Thomson *et al.* 1998; Tian *et al.* 2008). Compared to traditional full-length gene DNA vaccines, epitope-based DNA vaccines have advantages of increased ‘safety’ in that they are highly unlikely to integrate into the host genome; demonstrate ‘flexibility’ in epitope

selection, which should permit induction and optimisation of the desired type of immunity; have the potential to induce more potent immunoreaction than whole protein vaccines; and show the potential for induction of a broad spectrum of immune responses simultaneously targeting multiple antigens (Jackson *et al.* 2002). For instance, GG immunization of mice with a minigene coding for single epitopes derived from mutant p53 (an endogenous oncoprotein), or from HIV gp120 resulted in efficient induction of tumor protective CTL (Ciernik *et al.* 1996).

A polytope DNA plasmid containing multiple contiguous minimal murine CTL epitopes was developed. Protective CTL responses in immunized mice were demonstrated in recombinant vaccinia virus, influenza virus, and tumor challenge models. Moreover, CTL responses generated by this plasmid immunization lasted for one year. The ability to deliver large numbers of CTL epitopes using relatively small polytope constructs and DNA vaccination technology holds promise in the design of human epitope-based CTL vaccines, in particular, in vaccines against Epstein-Barr virus, HIV and certain cancers (Thomson *et al.* 1998).

IM injection of a multi-epitope DNA vaccine carrying three CTL epitopes of NP gene fused to the truncated HA from A/goose/Guangdong/1/96 (H5N1) did not produce haemagglutination inhibition (HI) antibody before challenge, but the percentages of $CD4^+$ and $CD8^+$ T lymphocytes in peripheral blood of immunized chickens increased significantly in comparison with chickens inoculated with a blank vector control. This vaccine also provided partial protection from homologous virus challenge (Peng *et al.* 2003).

1.4.4.3 DNA vaccines delivered by bacterial vectors

Some attenuated bacteria such as *Salmonella* spp. (Pan *et al.* 2009; Tacket *et al.* 2000), *Shigella* spp. (Fennelly *et al.* 1999; Sizemore *et al.* 1995) and *Listeria monocytogenes* (Frankel *et al.* 1995) are being used as delivery systems for plasmid DNA vaccines and as heterologous expression systems. Bacterial vectors can potentially be used via oral administration and may elicit mucosal immune response, but there are concerns regarding reversion to virulence of these bacteria and also whether preexisting immunity to the bacteria will preclude their use (Srivastava and Liu 2003).

1.4.4.4 Self-replicating genetic vaccines

In order to avoid the short intracellular half-life of RNA and its degradation by ubiquitous RNases, and to overcome the poor efficacy of some DNA-based and RNA-based vaccines, self-replicating replicons derived from the genomes of positive-stranded RNA viruses have been explored. Some alphaviruses, such as Sindbis virus (SIN), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE) are being developed as genetic vaccine vectors. In such a system, the structural protein region of the viral RNA genome is replaced with an antigen-encoding gene, while the enzymatic non-structural protein genes, known collectively as the viral "replicase," are retained. These replicon vectors can still direct their self amplification in an infected host cell and lead to high-level antigen expression (Chang *et al.* 2008; Dubensky *et al.* 2000; Vignuzzi *et al.* 2001).

A single IM injection of a SIN replicase-based vector expressing the glycoprotein B of HSV-1 induced virus-specific antibody and CTL responses, as well as protective

immunity in two different murine models. Moreover, 100- to 1,000-fold-lower doses of a SIN-based vector were required to induce comparable immune responsiveness to that achieved with a conventional plasmid DNA vector (Hariharan *et al.* 1998).

Mice immunized with a SFV-based vaccine expressing PR8 virus NP produced NP-specific antibody, a CTL response to NP epitopes and the level of protection from the replicon vector vaccine was comparable to that induced by plasmid DNA immunization (Vignuzzi *et al.* 2001).

1.5 Avian and human influenza DNA vaccines

Influenza DNA vaccines have been well studied with some of the earliest DNA vaccine development in mice conducted using influenza as a model (Ulmer 2002).

1.5.1 Plasmid vectors used in influenza DNA vaccines

Over the years various DNA expression vectors have been used in mice, chickens, or other animals as indicated below:

- The pcDNA 3.1 vector, a non-fusion vector containing CMV/IE promoter and neomycin resistance gene for cloning HA and M gene from A/chicken/Italy/1067/99 (H7N1) (Cherbonnel *et al.* 2003);
- The pCI vector, under the control of the CMV/IE promoter, for cloning HA gene from A/goose/Guangdong/1/96 (H5N1) (Chen *et al.* 2001);
- The pCI-neo vector, which contains theCMV/IE promoter, for cloning HA gene from A/turkey/Wisconsin/68 (H5N9) (Suarez and Schultz-Cherry 2000a);
- The pSI vector containing the SV40 early promoter, for cloning HA gene from A/turkey/Wisconsin/68 (H5N9) (Suarez and Schultz-Cherry 2000a);

- The pCAGGS/MCS vector, which contains the chicken β-actin promoter element and the SV40 origin of replication, for cloning full-length HA, NA, Ml, NP or NSl gene from PR8 virus (Chen *et al.* 2005; Chen *et al.* 1999a; Chen *et al.* 1998b) , HA and NA gene from A/chicken/Jiangsu/7/2002 (H9N2) (Qiu *et al.* 2006), HA gene from A/HK/156/97 (H5N1) (Kodihalli *et al.* 1999), HA gene from A/chicken/Victoria/1/85 (H7N7) and NP gene from A/turkey/Ireland/1/83 (H5N8) (Kodihalli *et al.* 2000); HA gene from A/turkey/Ireland/1/83 (H5N8) (Kodihalli *et al.* 1997), A/turkey/Wisconsin/68 (H5N9) (Suarez and Schultz-Cherry 2000a), and A/goose/Guangdong/1/96 (H5N1) (Jiang *et al.* 2007); In addition, HA, NA, M2, and NP genes from A/Hanoi/30408/2005 (H5N1) were cloned into pCAGα derived from a pCAGGS expression vector following a deletion of 829 bp between the *Eco*47III/*Xba*I sites (Patel *et al.* 2009);
- The VR1012 vector, which contains CMV/IE promoter, bovine growth hormone (BGH) polyadenylation site, and Kan^r gene in a modified pUC backbone, encoding NP and M gene from PR8 virus (Epstein *et al.* 2005; Epstein *et al.* 2000; Epstein *et al.* 2002); encoding HA from A/turkey/Wisconsin/68 (H5N9) (Suarez and Schultz-Cherry 2000a); VR10551, which is derived from VR1012 by replacing the BGH polyadenylation signal with a synthetic rabbit β-globin consensus polyadenylation signal, for cloning consensus NP, M1, and M2 sequences from human H3N2 and H1N1 influenza viruses (Jimenez *et al.* 2007), and HA, NP, and M2 gene of A/Vietnam/1203/04 (H5N1) virus (Lalor *et al.* 2008);

- The pRC/CMV vector, for cloning HA gene of A/WSN/33 (H1N1) influenza virus (Bot *et al.* 1997);
- The pRC/RSV vector, which contains the RSV long terminal repeat (LTR) promoter, for cloning HA from A/turkey/Wisconsin/68 (H5N9) (Suarez and Schultz-Cherry 2000a);
- The pHW2000 vector, containing the human pol I promoter and the murine terminator sequence which are flanked by a truncated CMV/IE promoter and by the BGH polyadenylation signal, for cloning HA gene from A/Hong Kong/1/68 (H3N2), A/Victoria/3/75 (H3N2), and A/Leningrad/360/86 (H3N2) (Huber *et al.* 2009);
- The pRSV vector, containing the RSV LTR promoter, the SV 40 virus t-intron, and the SV 40 polyadenylation site, for cloning NP gene from PR8 virus (Raz *et al.* 1994);
- The pI.17 vector, which is a pUC-based plasmid carrying a bacterial origin of replication, Amp^r gene, a truncated enhancer region, full promoter and full intron A gene from human CMV and a CMV terminator sequence, for cloning full-length HA gene of A/Sichuan/2/87 (H3N2) (Johnson *et al.* 2000);
- The pGA, which consists of CMV/IE promoter, BGH polyadenylation site, and Kan^r gene, for cloning NP or HA gene of PR8 virus (Sasaki *et al.* 2004);
- The pJW4303 vector, under the control of a CMV/IE promoter, for cloning HA, NP gene from PR8 virus (Feltquate *et al.* 1997; Pertmer *et al.* 2000), HA gene from A/turkey/Ireland/1/83 (H5N8) (Kodihalli *et al.* 1997);

- The pBC12/CMV/IL-2 vector, which contains CMV/IE promoter and SV40 origin of replication, rat preproinsulin II sequences (including an intron and a polyadenylation site), for cloning HA gene from PR8 and A/seal/Mass/1/80 (H7N7) (Fynan *et al.* 1993a);
- The V1J vector, which contains the CMV IntA enhancer, promoter, intron A for transcription initiation, the BGH terminator for transcription termination and ployadenylation in a modified pUC19 backbone, for cloning NP gene from PR8 (Bender *et al.* 1998; Deck *et al.* 1997; Montgomery *et al.* 1993; Ulmer *et al.* 1994; Ulmer *et al.* 1993; Ulmer *et al.* 1998);
- The pME18S vector, for cloning M and NP gene from PR8 (Okuda *et al.* 2001);
- The pRCAS, containing a nonpermuted proviral form of a replication-competent, derivative of the Schmidt-Ruppin-A strain of RSV (Hughes *et al.* 1987), and the p188, a defective-retroviral-based vector, for cloning HA gene of A/seal/Mass/1/80 (H7N7) (Robinson et al. 1993).

1.5.2 Effect of different influenza virus genes on protective immunity

DNA vaccination induced by different genes of AIV has been extensively explored and has shown promising, yet variable protective immune responses against homologous and heterologous AIV challenge. Cross-strain protective immunity against influenza based on CMI responses has been observed in mice, chickens and ferrets, and non-human primates as described in the following sections (Kodihalli *et al.* 2000; Laddy *et al.* 2008; Patel *et al.* 2009).

1.5.2.1 DNA vaccines encoding various viral genes in the murine model

1.5.2.1.1 HA-DNA

Plasmid DNAs encoding HA from influenza A viruses have been extensively investigated. A number of studies have demonstrated that immunization via parenteral, mucosal, or gene-gun delivery with plasmid DNA encoding HA from PR8 virus (Bender *et al.* 1998; Bot *et al.* 1997; Chen *et al.* 1999a; Chen *et al.* 1998b; Chen *et al.* 1999b; Deck *et al.* 1997; Fynan *et al.* 1993b; Montgomery *et al.* 1993; Pertmer *et al.* 2000; Robinson *et al.* 1997; Ulmer *et al.* 1994), A/Sichuan/2/87 (H3N2) (Johnson *et al.* 2000), A/HK/156/97 (H5N1) and A/turkey/Ireland/1/83 (H5N8) (Kodihalli *et al.* 1999), A/Hanoi/30408/2005 (H5N1) (Patel *et al.* 2009), A/seal/Mass/1/80 (H7N7) (Fynan *et al.* 1993b), and A/chicken/Jiangsu/7/2002 (H9N2) (Qiu *et al.* 2006) was able to produce cell-mediated and humoral immunity and protect mice against lethal challenge with homologous virus. Nonethless, a single dose of HA-DNA from PR8 virus administered by EP could not protect mice against homologous influenza virus challenge (Chen *et al.* 2005). Of note, immunization with plasmid DNA encoding A/HK/156/97 (H5N1) HA gene delivered via GG provided protective immunity against homologous A/HK/156/97 (H5N1) and antigenic variant A/Chicken/HK/258/97 (H5N1) viruses. However, plasmid DNA encoding HA gene from A/turkey/Ireland/1/83 (H5N8), which differs from A/HK/156/97 (H5N1) by 12% in the HA1 region at the amino acid level, prevented death but did not fully protect immunized mice against A/HK/156/97 (H5N1) challenge infection (Kodihalli *et al.* 1999). This highlights that AIV vaccines may induce limited cross-protection among the same HA subtypes. Moreover, immunization of mice via EP with a plasmid encoding synthetic consensus HA gene of H5N1 AIVs provided heterosubtypic protection against pathogenic human and H5N1 AIV infection (Laddy *et al.* 2008).

1.5.2.1.2 NP-DNA

As NP-DNA vaccines can induce immunity to conserved epitopes, it was hypothesized that such vaccines may protect the host against a broad range of viral variants (Epstein *et al.* 2000). Some studies showed that the plasmid DNAs encoding NP from PR8 virus failed to protect against challenge with homologous influenza virus (Chen *et al.* 1998b; Chen *et al.* 1999b; Pertmer *et al.* 2000; Robinson *et al.* 1997). Others have demonstrated that the plasmid DNAs encoding NP from PR8 virus induced CTL responses and partially protected mice from challenge with heterologous A/HK/68 (H3N2) influenza virus when dosed by GG or IM injection (Bender *et al.* 1998; Fu *et al.* 1997a; Montgomery *et al.* 1993; Ulmer *et al.* 1994; Ulmer *et al.* 1993; Ulmer *et al.* 1998). Intramuscular injection of a plasmid encoding NP gene from A/Hanoi/30408/2005 (H5N1) failed to protect mice against homologous virus challenge (Patel *et al.* 2009), while immunization of mice via EP with a plasmid encoding synthetic consensus NP gene of H5N1 AIVs provided heterosubtypic protection against A/Hanoi/30408/2005 (H5N1) challenge (Laddy *et al.* 2008). In particular, DNA priming-recombinant adenoviral boosting with NP generated strong antibody and T cell responses and hence conferred protection against challenge with multiple influenza A subtypes such as PR8, A/Philippines/2/82/X-79 (H3N2), H5N1 viruses A/HK/156/97 (H5N1) and A/HK/483/97 (H5N1) (Epstein *et al.* 2005).

Although the results of NP-DNA vaccination appear to be inconsistent in a number of studies, it did elicit NP-specific CTL responses in mice. Therefore, NP gene is being evaluated for potential development of broad spectrum influenza DNA vaccines.

1.5.2.1.3 NA-DNA

Mice vaccinated with NA-expressing DNA(NA-DNA) from PR8 virus by GG (Chen *et al.* 2005; Chen *et al.* 2000b; Chen *et al.* 1998b; Chen *et al.* 1999b), and NA-DNA from A/Aichi/2/68 (H3N2), A/Guizhou/54/89 (H3N2) (Chen *et al.* 2000b), and A/chicken/Jiangsu/7/2002 (H9N2) by EP (Qiu *et al.* 2006) showed significant protection against lethal challenge with the homologous virus. A single dose administration, even as low as 5 µg per mouse, of NA-DNA from PR8 virus by EP could protect mice from lethal challenge with homologous influenza virus and provided long-term protection against homologous virus infection (Chen *et al.* 2005).

Immunization with plasmid NA-DNA, prepared from A/Guizhou/54/89 (H3N2), A/Aichi/2/68 (H3N2) or PR8 virus by GG or EP, afforded protection or cross-protection against a homologous or variant virus infection within the same subtype, but failed to confer protection against a different subtype (Chen *et al.* 2000b). Intramuscular injection of a plasmid encoding NA gene from A/Hanoi/30408/2005 (H5N1) failed to protect mice against homologous virus challenge (Patel *et al.* 2009), while EP with a plasmid encoding synthetic consensus NA gene of H5N1 AIVs provided partial protection against A/Hanoi/30408/2005 challenge (Laddy *et al.* 2008). In comparison with HA-DNA and NP-DNA, although information that NA-DNA vaccine induced detectable antibodies and provided protection against influenza virus challenge was limited, NA may also be an effective viral component for the development of AI DNA vaccines (Chen 2004).

1.5.2.1.4 M-DNA and NS1-DNA

Due to the ongoing antigenic variation of influenza viruses, especially on the surface glycoproteins, efficacy of vaccines based on HA and NA can fluctuate over time. However, the viral matrix protein (M) protein is nearly invariant in all influenza A strains

and hence has potential as a broad spectrum immunogen (Ito *et al.* 1991; Slepushkin *et al.* 1995).

Mice given a DNA plasmid vaccine encoding M1 gene of PR8 virus were not protected against homologous challenge although they did produce a detectable antibody response (Chen *et al.* 1998b). Intramuscular injection of a plasmid encoding M2 gene from A/Hanoi/30408/2005 (H5N1) failed to protect mice against homologous virus challenge (Patel *et al.* 2009). However, a pME18S-M construct encoding M1 and M2 genes of PR8 virus provided 70% to 80% protection in the mice against challenge with not only homologous (PR8) but also heterologous (A/WSN/33) (H1N1) influenza viruses (Okuda *et al.* 2001). When this vaccine was applied topically together with cholera toxin or a synthesized CpG ODN as adjuvant, it conferred cross-protection against challenge with A/Udorn/72 (H3N2) virus (Ozaki *et al.* 2005). These studies suggested that M-DNA can provide some cross-protective immunity and this is being considered further in relation to the development of broad spectrum influenza vaccines. Alternatively, in mice administered plasmid encoding NS1 gene (NS1-DNA) there was no evidence of protection after homologous challenge (Chen *et al.* 1998b).

1.5.2.1.5 Co-administration of mixed plasmids encoding different genes

Promising results with some of the single gene-expressing DNA vaccines against influenza A viruses in mice encouraged further investigations using combinations of genes especially to produce broader spectrum protection. The following combinations have been investigated.

1.5.2.1.5.1 NP-DNA and M-DNA

BALB/c mice dosed with a mixture of NP-DNA and M-DNA from PR8 virus gave a modest reduction in virus replication in lung which showed some correlation with improved survival after homologous challenge compared with unvaccinated controls (Epstein *et al.* 1997; Levi and Arnon 1996). Mice vaccinated with NP-DNA and M-DNA combination survived after lethal challenge with A/Philippines/2/82/X-79 (H3N2) influenza virus whereas B6-β2m (-/-) mice (defective in normal MHC class I complexes) that received identical vaccination died following lethal virus challenge (Epstein *et al.* 2000).

Vaccination of a mixture of NP-DNA and M-DNA from PR8 virus reduced replication of A/Hong Kong/486/97 virus (a nonlethal H5N1 strain) in the lungs compared to controls, protected mice against lethal challenge with A/Hong Kong/156/97 (intermediate virulence), but failed to protect against challenge with A/Hong Kong/483/97 (highly virulent strain) challenge. Additionally, all mice primed with a mixture of NP-DNA and M-DNA and subsequently exposed to A/Hong Kong/156/97 challenge survived after re-challenge with A/Hong Kong/483/97 (Epstein *et al.* 2002).

Compared to single plasmid vaccines expressing NP, M1, M2, or other combinations of them from consensus sequences of human H3N2 and H1N1 influenza viruses, intramuscular injections of a cationic lipid-formulated NP-DNA and M2-DNA plasmid combination gave the highest level of protection against lethal challenge with A/HK/8/68 (H3N2) or PR8 viruses in mice (Jimenez *et al.* 2007).

A Vaxfectin-formulated vaccine containing NP-DNA and M2-DNA derived from highly pathogenic A/Vietnam/1203/04 (H5N1) influenza virus strain gave significant protection against death in mice and provided some protection in ferrets (Lalor *et al.* 2008).

Studies with plasmids encoding NP and/or M gene of H1N1, H3N2 or H5N1 influenza viruses suggested that immune responses against conserved internal proteins were likely to provide some protection and could play a role in decreasing influenza transmission (Epstein *et al.* 2002; Lalor *et al.* 2008).

1.5.2.1.5.2 Various combinations of HA-DNA, NA-DNA, M-DNA and NP-DNA

Gene gun administration of plasmid DNA combinations either HA-DNA+ NA-DNA or HA-DNA + NA-DNA + M1-DNA, respectively encoding HA, NA and M1 genes from PR8 virus resulted in almost complete protection of mice against homologous virus challenge, whereas, mice that received either HA-DNA or NA- DNA alone showed only partial protection against challenge and mice given M1-DNA failed to show any protection. Moreover, both of the plasmid DNA mixtures (HA-DNA + NA-DNA) and (HA-DNA + NA-DNA + M1-DNA) exhibited a slight tendency to confer cross-protection against an A/Yamagata/120/86 (H1N1) virus challenge, a drift mutant of the same virus subtype. The addition of the M- DNA to HA-DNA+ NA-DNA mixture did not appear to strengthen the degree of protection (Chen *et al.* 1999a). In another study, one-day-old or 12-week-old mice administered a combined HA-DNA and NP-DNAs from PR8 virus provided slightly better protection against the lethal PR8 challenge than HA-DNA or NP-DNA alone (Pertmer *et al.* 2000).

Intramuscular immunization of a Vaxfectin-formulated vaccine containing consensus HA, NP, and M2-expressed plasmids from A/Vietnam/1203/04 (H5N1) conferred complete protection from lethal homologous H5N1 virus challenge in mice and ferrets (Lalor *et al.* 2008).

As compared to vector-immunized controls, EP with combined DNA vaccines respectively encoding consensus HA, NP and NA genes of H5N1 AIVs provided protection against A/Vietnam/1203/2004 (H5N1) challenge, in terms of weight loss and reduction in viral load in ferrets (Laddy *et al.* 2008) and in terms of reduction in viral loads, and absence of histopathological changes in lungs (inflammation, infiltration, and edema) in rhesus macaques (Laddy *et al.* 2009).

In summary, protection studies of immunized mice challenged with homologous or heterologous influenza A viruses indicate that HA afforded better protection than the NA, NP or M2 DNA vaccines (Patel *et al.* 2009). Co-administration of mixed plasmids provides a better immune response than single plasmids alone. Inclusion of conserved NP and M2 influenza antigens in a HA-expressing plasmid vaccine may increase the cross-protective antiviral response. Thus, DNA vaccination with conserved influenza genes may provide an alternative in the control of a rapidly spreading pandemic virus in the absence of antigenically matched HA-based vaccines (Epstein *et al.* 2002; Lalor *et al.* 2008).

1.5.2.2 DNA vaccines encoding various avian influenza genes in the chicken model

In contrast to the extensive body of literature relating to DNA vaccines for influenza viruses in the murine model, relatively little is reported on DNA vaccines against AIVs in the chicken model. Available information is described below.

1.5.2.2.1 HA-DNA

DNA vaccines encoding HA gene from A/goose/Guangdong/1/96 (H5N1) (Chen *et al.* 2001), A/turkey/Ireland/1/83 (H5N8) (Kodihalli *et al.* 1997; Kodihalli *et al.* 2000),

A/turkey/Wisconsin/68 (H5N9) (Suarez and Schultz-Cherry 2000a), A/chicken/Italy/1067/99 (H7N1) (Cherbonnel *et al.* 2003), A/seal/Mass/1/80 (H7N7) (Fynan *et al.* 1993a; Fynan *et al.* 1993b; Robinson *et al.* 1993), A/chicken/Victoria/1/85 (H7N7) (Kodihalli *et al.* 2000), A/chicken/China/N/2005 (H9N2) (Pan *et al.* 2009) have been reported and these vaccines gave variable levels of protective immunity against lethal homologous virus challenge in chickens.

Gene gun administration of HA-DNA from A/turkey/Ireland/1/83 (H5N8) provided 95% cross-protection against challenge with lethal antigenic variants that had 11 to 13% amino acid sequence difference in the HA1 region from A/turkey/Ireland/1/83 virus (Kodihalli *et al.* 1997). DNA vaccines encoding HA gene either from A/turkey/Ireland/1/83 (H5N8) or A/chicken/Victoria/1/85 (H7N7) administered via GG gave over 85% protection against homologous virus challenge, with minimal virus shedding in tracheal and cloacal swabs. However, both plasmids failed to elicit a cross-protective immunity against different subtypes of challenge virus (Kodihalli *et al.* 2000).

Plasmid DNA vaccines have been considered as alternatives to inactivated influenza virus vaccine and fowlpox-vectored vaccines (Zheng *et al.* 2009). Studies showed that H1-, H3-, H5-, H7- and H9-encoding DNA vaccines have protected mice and chickens from homologous influenza A virus challenge (Chen *et al.* 1998b; Cherbonnel *et al.* 2003; Huber *et al.* 2009; Laddy *et al.* 2007; Qiu *et al.* 2006). Some protection was also shown against challenge with heterologous viruses within the same subtype but not against different subtypes. To date there have been no reports on the efficacy of H6-HA DNA vaccines in mice or chickens.

1.5.2.2.2 NP-DNA

Gene gun delivery of plasmid encoding NP gene from A/turkey/Ireland/1/83 (H5N8) reduced the severity of infection and conferred 50% protection on chickens from homologous challenge and 42% protection against heterologous A/chicken/Victoria/1/85 (H7N7) virus challenge, suggesting that this NP-DNA vaccine did not induce substantial levels of protective immunity against either homologous or heterologous AIVs (Kodihalli *et al.* 2000).

1.5.2.2.3 Combined plasmids

As with DNA vaccines in mice, combination plasmid vaccines have also been used in chickens to investigate if this improved vaccine efficacy. Chickens inoculated via GG with a combination DNA vaccine consisting of two plasmids respectively encoding the HA genes from A/turkey/Ireland/1/83 (H5N8) or A/chicken/Victoria/1/85 (H7N7) acquired complete protection against challenge with the respective viruses and the vaccinated chickens showed no virus shedding in tracheal and cloacal swabs (Kodihalli *et al.* 2000). Immunization of chickens with a combination of HA-DNA and M-DNA plasmids respectively encoding HA and M gene from A/chicken/Italy/1067/99 (H7N1) using the Medijector device appeared to induce higher HI antibody titre than HA-DNA alone and gave improved protection against homologous challenge (Cherbonnel *et al.* 2003).

1.5.2.3 DNA vaccine encoding various influenza virus genes in other animal models

African Green monkeys injected with a mixture of five DNAs encoding HA gene from A/Beijing/353/89 (H3N2), A/Texas/36/91 (H1N1), and B/Panama/45/90 and NP and M1

from PR8 virus induced HI antibodies to A/Beijing/353/89. Ferrets immunized with DNA encoding HA from PR8 virus and A/Georgia/03/93 (H3N2) also elicited HI antibodies and they were protected against homologous virus challenge (Donnelly *et al.* 1995a).

Plasmid DNA vaccines encoding HA from A/Hawaii/01/91 (H3N2), and NP and Ml from A/Beijing/353/89 (H3N2) were investigated in ferrets and shown to provide protection against challenge with a divergent, drift variant virus A/Johannesburg/33/94 (H3N2) and the extent of protection conferred by the DNA vaccine was comparable to that provided by an inactivated A/Johannesburg/33/94 vaccine (Donnelly *et al.* 1997a).

Itradermal immunization of pigs with an M2eNP DNA construct encoding NP gene of A/swine/Oedenrode/96 (H3N2) virus and M2 gene from A/swine/Best/96 (H1N1) virus induced an antibody response to M2 protein but failed to confer protection against A/swine/Best/96 (H1N1) virus challenge. Unexpectedly, the M2eNP DNA-vaccinated pigs displayed more severe clinical signs than were expected from challenge by this virus (Heinen *et al.* 2002).

Immunization of rabbits via GG with a plasmid encoding codon optimized HA or truncated HA genes from A/NewCal/20/99 (H1N1) or A/Panama/2007/99 (H3N2) influenza virus elicited strong HI and neutralizing antibody responses (Wang *et al.* 2006).

Intradermal and mucosal vaccination of ponies via GG with a plasmid DNA encoding HA gene A/equine/Kentucky/1/81 (H3N8) conferred complete protection from challenge with homologous virus infection, while ID vaccination alone only afforded partial protection (Lunn *et al.* 1999).

1.5.3 Specific aspects of the humoral immune response to avian influenza DNA vaccines

Anti-HA antibody induced by plasmid DNAs encoding HA from AI virus strains played a key role in neutralizing virus and producing protective efficacy in mice, chickens, ferrets and nonhuman primates against challenge with homologous and heterologous HPAI viruses (Kodihalli *et al.* 1997; Laddy *et al.* 2008).

1.5.3.1 Antibody response in chicken model

The protocols that have been used for DNA vaccination in chickens are similar to those used in the murine model. The minimal and maximal doses of plasmid DNA used per bird have varied between 50 µg (Chen *et al.* 2001; Cherbonnel *et al.* 2003) to 300 µg (Fynan *et al.* 1993a) for saline-DNA injection, and from 0.4 ng (Fynan *et al.* 1993b) to 10 µg (Kodihalli *et al.* 2000) for GG delivery. A few studies have employed a single vaccination (Feltquate *et al.* 1997), but most studies utilized booster immunizations (Fynan *et al.* 1993a; Jiang *et al.* 2007; Kodihalli *et al.* 1997; Robinson *et al.* 1993). Dosage intervals between vaccinations of two weeks (Chen *et al.* 2001), three weeks (Cherbonnel *et al.* 2003; Jiang *et al.* 2007; Le Gall-Recule *et al.* 2007; Suarez and Schultz-Cherry 2000a) and four weeks (Fynan *et al.* 1993a; Fynan *et al.* 1993b; Kodihalli *et al.* 1997; Lee *et al.* 2006; Robinson *et al.* 1993) have been successfully used.

Various delivery routes for DNA inoculation have been examined in chickens and the effect of DNA delivery routes on protective immunity has been assessed. Intramuscular, IV and intratracheal administration of a H7-expressing DNA vaccine provided better protective immunity against lethal A/chicken/Victoria/1/85 (H7N7) challenge than SC, IP, intrabursal, and intraorbital inoculations (Fynan *et al.* 1993b). Nevertheless, immunization with 100 µg of a H7-expressing plasmid by each of three routes (IM, IP

and IV) followed by boosting with 300 μg DNA (100 μg IV, 100 μg IP, and 100 μg IM) showed that IM delivery did not improve the level of protection in comparison with the trials which did not include the IM route (Fynan *et al.* 1993a). Gene gun delivery of a H5-expressing plasmid conferred complete immune protection against lethal H5 virus challenge, but as discussed previously this delivery system is not practical in the chicken industry (Kodihalli *et al.* 1997).

1.5.3.1.1 Anti-HA antibody

Unlike immune response induced by HA-DNA in mice, some studies in chickens have demonstrated only low to undetectable levels of HA specific antibodies resulting from primary and booster immunization with DNA vaccine, although high antibody titres resulted from challenge with AI virus (Fynan *et al.* 1993a; Kodihalli *et al.* 1997; Kodihalli *et al.* 2000; Suarez and Schultz-Cherry 2000a). However, studies in chickens have also reported that other DNA vaccines generated acceptable antibody responses (Chen *et al.* 2001; Cherbonnel *et al.* 2003; Jiang *et al.* 2007; Suarez and Schultz-Cherry 2000a).

1.5.3.1.2 Anti-NP antibody responses

Similar to the findings of poor HI antibody responses after the use of HA-expressing plasmids in some studies, chickens immunized via GG with a plasmid encoding the NP gene from A/turkey/Ireland/1/83 (H5N8) failed to induce detectable antibodies to NP as measured by ELISA. Following challenge with homologous influenza A/turkey/Ireland/1/83 (H5N8) virus or heterologous influenza A/chicken/Victoria/1/85 (H7N7) virus, some birds survived and they produced high levels of HI antibodies to the challenge viruses (Kodihalli *et al.* 2000).

1.5.3.2 Antibody response in other animal models

Immunization via EP with a combination of synthetic consensus plasmid DNAs encoding HA, NP and NA of H5N1 AIVs induced marked cross-reactive antibodies to divergent H5N1 viruses in ferrets (Laddy *et al.* 2008) and rhesus macaques (Laddy *et al.* 2009).

1.5.4 Specific aspects of CMI response to avian influenza virus DNA vaccines

Due to ongoing antigenic shift and drift of influenza A virus, strain-specific antibody response produced by protein-based vaccines do not remain effective and need to be regularly updated. For this reason intensive attention has focused on investigation of vaccines to induce CMI response which may have the potential to induce cross-protection against homosubtypic and heterosubtypic influenza A viruses. Although each of the viral proteins (including HA, NA) can trigger a CTL response, the primary target of CMI responses typically focus on peptides from internal conserved proteins of the influenza A virus (Kodihalli *et al.* 2000; Laddy *et al.* 2008; Thomas *et al.* 2006).

DNA vaccines encoding conserved antigens have been shown to protect against diverse influenza A subtypes in mice (Bender *et al.* 1998; Epstein *et al.* 2005; Epstein *et al.* 2002; Ulmer *et al.* 1993; Ulmer *et al.* 1998), chicken (Kodihalli *et al.* 2000) and ferrets (Donnelly *et al.* 1995a). This protection is mediated by both class I-restricted CTLs and Th1-type cytokine secreting helper T cells (Ulmer *et al.* 1993; Ulmer *et al.* 1998).

The CTL response to NP is characterized by cross-reactivity between various subtypes of influenza A viruses and there is reduced sensitivity to antigenic drift with this viral protein (Kodihalli *et al.* 2000; Shu *et al.* 1993). Long-lasting anti-NP CTL activity has been observed *in vivo* (Raz *et al.* 1994; Yankauckas *et al.* 1993). In addition, CMI

responses induced by DNA vaccines were not inhibited by the presence of maternal antibody (Pertmer *et al.* 2000).

Because many reagents defining surface markers on important cellular population are of murine origin, and numerous recombinant and congenic mouse strains, and more recently transgenic and knockout strains are available, much of the work on the celluar immunity to influenza A virus has been extensively conducted in mice as described in section 1.4.1.3 (Epstein 2003). In contrast, due to unavailability of monoclonal antibodies (mAbs) recognizing avian T cell-associated antigens as well as the very limited number of inbred lines of chickens with genetically defined MHC haplotypes, knowledge of the specifics of CMI against AIV in avian species is limited (Kapczynski 2008).

Despite the absence of detectable antibodies to NP following immunization with the plasmid pCMVNP from A/turkey/Ireland/1/83 (H5N8), 42-50% chickens survived following homologous and heterologous virus challenge (Kodihalli *et al.* 2000). This partial protection may relate to the CMI activation and mobilization, which became functional in half of the chickens before the highly pathogenic H5 or H7 virus infection was well established and caused fatalities. It is also possible that cytokines like IFN-γ production from virus specific T-memory cells, as observed by studies with NP-DNA in a mouse model (Bot *et al.* 1998), were effective in shutting virus infection down in half of the chickens before overwhelming H5 or H7 HPAI infection occurred. Unfortunately, cytokine measurement was not conducted in that study. In another chicken study using multi-epitope DNA vaccines against H5N1 AIV, $CD4^+$ and $CD8^+$ T lymphocyte in the peripheral blood of immunized chickens were reported to increase significantly in comparison to a blank vector control (Peng *et al.* 2003). In addition, real-time PCR

testing showed that the mRNAs for nine cytokines (IFN-α, IFN-β, IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-15, and IL-18) were expressed in HPAI virus infected chickens, showing that HPAI infection quickly induced intensive antiviral and proinflammatory cytokine mRNA expressions in chickens as compared with a LPAI strain (Suzuki *et al.* 2009). In addition, *in vivo* EP of a plasmid expressing consensus NP protein of H5N1 AIVs elicited IFN- γ-based CMI responses in the rhesus macaque model (Laddy *et al.* 2009).

1.5.5 Protective efficacy related to both cellular and humoral immune responses to H5N1 avian influenza viruses

In a study on evaluation of DNA vaccines encoding conserved or variable influenza antigens against different isolates of H5N1 viruses in mice, the T cell response, as analyzed by ELISpots, induced by the HA-expressing plasmid was higher than those from those plasmids encoding NA, NP or M2 gene. The T cell responses were also directly proportional to the level of protection, with HA-expressing plasmid > NA- > NP- > M2- expressing plasmids. Despite the absence of a detectable B cell response, as measured by HI and neutralizing antibody, a T cell response was still detected after vaccination with low doses of HA-expressing plasmid and this was capable of giving protection (Patel *et al.* 2009).

In another study in mice, ferrets and non-human primates, plasmids encoding synthetic consensus HA, NP and NA gene of H5N1 AIVs were administered via EP. In mice, the HA-DNA vaccine provided complete protection against A/Hanoi/30408/2005 (H5N1) virus challenge, and there was partial protection shown by the NP-DNA vaccine and NA-DNA vaccine, but this was greater for the NP-DNA vaccine than for the NA-DNA vaccine. With regards to CMI response, all three plasmids induced IFN-γ-based cellular

immune responses in mice, largely dependent upon $CD8^+$ T cells, with responses to NP> HA > NA. In terms of antibody response, HA-DNA elicited a strong antibody response in mice, as determined by ELISA and HI assays whereas neither NA-DNA nor NP-DNA induced detectable antibody responses in mice. In ferrets and rhesus macaques, the combination of HA-DNA, NP-DNA and NA-DNA induced strong antigen-specific CMI responses and cross-reactive antibody responses to HA. Despite the absence of NP antibody response NP-DNA induced potent CMI responses and conferred a statistically significant degree of protection with reduction in average weight loss (Laddy *et al.* 2008).

In another study, single immunization of mice with a plasmid expressing HA gene of A/Chicken/Henan/12/2004 (H5N1) induced acceptable levels of circulating anti-influenza virus antibodies and also a potent systemic CMI response with high level of IFN-γ-producing cells and low levels of IL-4-producing cells (Zheng *et al.* 2009).

Taken together, plasmid DNA-induced protective immunity involves interaction between B cells and T cells (either $CD4^+$ or $CD8^+$ cells). It is necessary to assess the relationship between both arms of the plasmid-induced immune responses in evaluating DNA vaccines with regards to protective efficacy against influenza A virus (Patel *et al.* 2009).

1.6 Hypotheses of the thesis

1. That H6-DNA vaccines are immunogenic and can induce a protective immune response in vaccinated chickens against specific H6N2 AI viruses.

2. That enhancement of DNA vaccines in chickens against AIVs can be achieved by approaches such as selection of DNA expression vectors, incorporation of enhancer

sequences, codon optimization of the HA gene, optimization of the dose of injected plasmid DNA and the number of immunizations.

3. That antibody responses to AI DNA vaccines can be further enhanced by different administration methods, and by the use of adjuvants including lipofectin and different chemical adjuvants, including a novel nanoparticle, Phema.

Chapter 2

Biological and genetic characterization of the H6N2 influenza virus originating from a healthy Eurasian coot in Western Australia

2.1 Introduction

Avian influenza virus (AIV) infection in poultry can exhibit various signs from an asymptomatic infection to an acute, fatal disease (Lee *et al.* 2007). Two surface glycoproteins, haemaggglutinin (HA) and neuraminidase (NA), responsible for attachment to cell receptors to initiate infection and the release of virus particles from host cell receptors respectively, are the most important protective components among the viral proteins (Hampson 2006). Internal nucleoprotein (NP), related to the switching of viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis, is a major target of the host cytotoxic T-cell immune response (Tamura *et al.* 2005; Yewdell *et al.* 1985).

Waterfowl are commonly believed to be a major reservoir of the type A influenza viruses which are antigenically and genetically diverse (Slemons *et al.* 1991; Slemons and Swayne 1995). These waterfowl-origin viruses are occasionally associated with outbreaks of severe avian influenza in domestic poultry, characterised by swollen kidneys and visceral urate deposits (Slemons and Swayne 1990, 1995). Chickens and turkeys experimentally challenged with waterfowl-origin type A influenza viruses became infected but remained clinically normal, even though high virus titres could be detected in kidney tissues and viral nucleoprotein was detectable in renal tubular epithelial cells and in intestinal mucosal epithelial cells, suggesting that these isolates could be nephrotropic and enterotropic (Slemons and Swayne 1990, 1995). More recently, there were reports on H6 subtype AIV outbreaks in commercial poultry (Abolnik et al. 2007; Woolcock et al.

2003; Yee et al. 2009). This highlighted that waterfowl-origin virus can present a potential threat to domestic poultry.

The H6 subtype AIV was first isolated from a turkey in 1965, and other H6 viruses were subsequently isolated from shorebirds and wild ducks (Downie *et al.* 1977; Downie and Laver 1973; Sharp *et al.* 1993). The H6 subtype influenza viruses have received little attention until the first documented infection of humans with an H5N1 AIV occurred in Hong Kong in 1997. An H6N1 influenza virus, A/teal/HK/W312/97 (H6N1) isolated during this outbreak was found to have the same internal gene and NA gene segments as this A/HK/156/97 (H5N1) virus. This indicated that this isolate seemed to be one progenitor of the A/HK/156/97 (H5N1) virus (Hoffmann *et al.* 2000). Although natural human infection with H6 subtype has not yet been reported, a recent seroprevalence study showed that United States veterinarians who have been exposed to birds demonstrated significantly elevated antibody titers against H5, H6, and H7 AIVs (Myers *et al.* 2007). It raised the possibility that H6 viruses could become novel human pathogens (Chin *et al.* 2002).

In addition, H6 viruses are one of the most commonly recognized subtypes in domestic ducks in southern China (Cheung et al. 2007) and in migratory birds in North America and Europe (Munster et al. 2007; Spackman et al. 2005). Surveillance has demonstrated that the continued co-circulation of H5N1, H6N1, and H9N2 AIVs in southern China has led to frequent reassortment in minor poultry species (Cheung et al. 2007; Chin et al. 2002). This in turn greatly increases the genetic diversity of influenza A viruses in this region and highlights the potential for H6 viruses and H6 reassortants to cross the species barrier to infect humans. Understanding the epidemiology, ecology and evolution of H6

viruses will be helpful to further understand the molecular evolution of H5N1 AIV and may help in the development of future control measures.

The objective of the work described in this chapter was to provide baseline information on the biological and genetic properties of an H6N2 AIV isolate (A/coot/WA/2727/79), which was isolated from a healthy Eurasian coot (*Fulica atra*) in Western Australia (Mackenzie *et al.* 1984), as well as the potential pathogenicity before its use as a model virus for a proof-of-concept DNA vaccine research.

2.2 Materials and Methods

2.2.1 H6N2 avian influenza virus, replication and identification

Avian influenza virus A/coot/WA/2727/79 (H6N2) was kindly provided by Prof. John Mackenzie, University of Western Australia, Australia from virus stocks held at the Animal Virology laboratory, DAFWA. The virus stock was propagated at DAFWA using 9-11 day-old specific pathogen-free (SPF) chicken embryonated eggs (White Leghorn, SPAFAS Inc., VIC, Australia) according to the standard method provided by the World Organisation for Animal Health (OIE)(OIE 2008). Briefly, 0.1 mL volumes of 10-fold dilutions of virus stock in sterile PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO4 1.15 g, KH_2PO4 0.2 g, add distilled water (dH_2O) up to 1L, pH7.4) were inoculated into the allantoic cavity of 9-11day-old embryonated chicken eggs. The eggs were incubated at 35–37°C for 4–5 days. Following chilling at 4°C overnight, the allantoic fluid of these eggs was harvested and tested for haemagglutination (HA) activity. The stock virus was passaged two additional times to compare HA titres after passage. Individual aliquots of HA positive allantoic fluid were also tested to ensure bacterial sterility before pooling. The pooled HA positive virus was tested to confirm presence of H6 activity by a

haemagglutination inhibition (HI) test using in-house H6 reference positive sera from DAFWA. The virus with HA activity was stored at -70°C as working stocks for later experiments. This new viral stock, amplified in SPF or specific antibody negative (SAN) chicken embryonated eggs (Altona Hatchery Pty. Ltd., WA, Australia) from 1-2 passages, was used to conduct infectivity titrations in chicken embryos, provide antigen for inactivated vaccine preparation and challenge virus for subsequent studies.

Confirmatory H subtyping of A/coot/WA/2727/79 (H6N2) was performed at Tai Lung Veterinary Laboratory, AFCD, Hong Kong. The reference H6 antiserum to A/Ost/RSA/946/98 (H6N8) virus was provided by Veterinary Laboratories Agency, Weybridge, Surrey, UK. The HI test was performed as per the OIE standard protocol. The HI titre against 4 HA units of inactivated virus antigen was 1:160, which was consistent with other H6 viruses in the collection at Tai Lung Veterinary Laboratory. The N subtype of this H6N2 virus was identified as N2 subtype by neuraminidase inhibition testing at AAHL, Australia. This information was provided by Dr Paul Selleck at AAHL.

2.2.2 HA antigen preparation

A/coot/WA/2727/79 (H6N2 was amplified by inoculating 0.1 mL virus (approximately 10^3 EID_{50}) into the allantoic cavity of 10-day-old SAN eggs and harvesting allantoic fluid from the eggs on day 5. Following confirmation of HA activity by a spot HA test, the infectious allantoic fluids were harvested and clarified by centrifugation. The virus was inactivated by adding a final concentration of 0.1% (v/v) formaldehyde (37% formalin, Sigma, MO, USA) and stirring the allantoic fluid for 20 hr at 37°C. Effectiveness of inactivation was evaluated by inoculating 0.2 mL of undiluted and 1/10 diluted allantoic fluid respectively into each of five 10-day-old eggs, incubating for 1 week, and testing

allantoic fluid for HA activity. The completely inactivated allantoic fluid was aliquotted and stored at -20°C until required.

2.2.3 Haemagglutination (HA) and Haemagglutination Inhibition (HI) tests

2.2.3.1 Preparation of 0.5% chicken red blood cells (CRBC)

The SPF or SAN chicken blood was collected and mixed with an equal volume of Alsever's solution (citric acid 0.055 g, sodium citrate 0.8 g, D-Glucose 2.05 g, NaCl 0.42 g, add dH_2O to make up to 100 mL; sterilize by autoclaving at 116°C for 10 min). The blood was centrifuged in a graduated conical tube at 800 g for 5 min. Following removal of the supernatant and the buffy coat, the red cells were resuspended in PBS, and mixed with a Pasteur pipette gently, before a further centrifugation step for 5 min at 800 g. This process was repeated twice. The red cell pellet was diluted to a concentration of 0.5% (packed cell v/v) in PBS containing 0.1% foetal calf serum (FCS) (Gibco, Melbourne, Australia).

2.2.3.2 HA test

HA assays were performed as per the OIE procedure (OIE 2008). Briefly, 25 µL volumes of PBS were aliquotted across a 96-well U plate (Greiner Bio-One, Frickenhausen, Germany). Then 25 µL of antigen was added to the first well and two-fold serial dilution was made across the plate to the last well. Finally 25 µL of PBS and 25 µL of 0.5% CRBC were added to all wells and the plate was shaken on the plate shaker. The plate was incubated at room temperature for 40 min before being examined for HA activity. The endpoint was the highest dilution at which complete hemagglutination occurred and this was considered as 1 HA unit. The HA titre of the antigen was then calculated.

2.2.3.3 HI test

HI assays were performed as per the OIE procedure (OIE 2008). In brief, 25µL of PBS was added across to all wells of a 96-well U-bottomed plate. Then 25µL of serum was added to the first well, and two-fold serial dilutions were made across the plate to the last well, with the last 25µL mixture being discarded. Finally, 25µL of antigen (containing 4 HA units) was added to all diluted serum wells. The plate was mixed briefly and then incubated at room temperature for 30 min to 60 min, before 25 µL of 0.5% CRBC was added to the wells. After shaking, the plate was incubated at room temperature for a further 40 min until a positive haemagglutination reaction developed in non-serum containing control wells. The HI titre was the reciprocal of the highest serum dilution fully inhibiting haemagglutination.

The same procedure was used with H6 reference antiserum to determine the presence of H6 virus in inoculated allantoic fluid.

2.2.4 Cell culture for virus growth

2.2.4.1 Cell passage, storage and resuscitation

Madin-Darby Canine Kidney cells (MDCK), provided by DAFWA, were grown in 25 or 75cm^2 plastic flasks (Nalge, Nunc International, Denmark) in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Auckland, New Zealand)(make up 1 packet (~10g) powdered medium to 1L and 23.8 mM $NaHCO_3$, filter-sterilised) supplemented with 10% (v/v) FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C in a 5% CO_2 humidified incubator. When monolayers were confluent in the flask, the media was removed and the cells were washed with sterile PBS, then 5

mL of 0.25% trypsin (Difco, NJ, USA), 1 mM EDTA, Hank's balanced salt solution (HBSS, Invitrogen, Melbourne, Australia) were added to the flask and incubated for 2-5 min at 37°C to dislodge the cells from the plastic. The cells were passaged in DMEM containing 10% FCS, using a split ratio of 1:3, and incubated in flasks at 37°C in a 5% CO_2 humidified incubator, or maintained in DMEM with 2% FCS.

Cells in monolayers were washed and trypsinised during mid-log phase of growth. DMEM containing 10%FCS was added and the cell suspension was centrifuged at 1000g for 5 min at 4 °C. The supernatant was removed and the pellet was gently resuspended in 5mL DMEM containing 20% FCS. An equal volume of DMEM containing 20% DMSO was added very slowly. The cell suspension was then aliquoted into 1mL cryovials (Nunc International), and stored at 4°C for 2 hr, followed by -80°C overnight. Subsequently, the cryovials were placed into liquid nitrogen for long term storage.

Once a cell cryovial was taken out from liquid nitrogen it was thawed rapidly in a container with 37-40°C water. Five milllitres of pre-warmed DMEM with 10% FCS was slowly added to the cells. Cells were then centrifuged at 1,000g for 5 min and resuspended in 10 mL DMEM with 10% FCS. The cells were placed into a flask followed by incubation at 37°C with 5% CO_2 for a few days to form a monolayer.

2.2.4.2 Virus propagation

Virus growth in MDCK cells was performed according to the standard WHO protocol (WHO 2002).Cells were propagated in 25 cm^2 tissue culture flasks (T-25 flask, BD Biosciences, NSW, Australia) or 6-well culture plates (Nunc International). When the MDCK cells formed a monolayer, the medium was discarded and the cells were washed

three times with 6 mL (flask) or 2mL (6-well plate) of virus growth medium consisting of DMEM supplemented with antibiotics, L-glutamine, 0.2 % bovine serum albumin fraction V (Gibco BRL), with or without the addition of 2 μg/mL TPCK-trypsin (Sigma, MO, USA). Aliquots of 100 μl undiluted (for T-25 flask) or 1/10 diluted (plate) infective allantoic fluid virus were inoculated into the monolayer cells. The inoculum was adsorbed for 30 min at 37°C followed by the addition of 6mL (flask) or 2mL (plate) of virus growth medium with or without 2 μg/mL of TPCK trypsin. The cells were observed daily for 1 week for a cytopathic effect (CPE). CPE was confirmed to be virus specific by HA and immunofluorescent testing.

2.2.5 Immunofluorescent Test (IFT)

MDCK cells were propagated on microscope cover slips in a 6-well plate, and inoculated with H6 virus. At 36-72 hr post-inoculation the medium was removed. Fixing solution, consisting of 2 mL of ice cold methanol/acetone (1:1) was added into the 6-well plate and left at room temperature for 10 min. The plate was then stored at 4°C overnight or at -20°C for long term storage. Following air-drying, the plate was washed once with PBS before 1 mL anti-H6 serum (made from chickens vaccinated with inactivated whole H6N2 virus) with a dilution of 1:100 was added and incubated at 37°C for 45 min. The plate was washed three times for 5 min each using PBST (0.05% Tween-20 in PBS). This was followed by the addition of 0.5 mL fluorescein isothiocyanate (FITC)-conjugated anti-chicken immunoglobulin G (IgG) at a dilution of 1:250 (Sigma). After incubation at room temperature for 30 min, the plate was washed three times with PBST. The cover slip was inverted onto a glass slide and observed for fluorescent staining under a fluorescent microscope (Olympus BX50, Tokyo, Japan).

2.2.6 Infectivity titration of the virus

Serial 10-fold dilutions of A/coot/WA/2727/79 (H6N2) virus stock were made using PBS. Aliquots of 0.1 mL of diluted fluid were inoculated into the allantoic cavity of five embryonated eggs of between 9 days and 11 days of age, or onto monolayers of MDCK cells in 96-well plates. Embryos or plates were incubated at 37°C for 7 days and observed daily to check for mortalities or CPE. The allantoic fluid of each egg or culture medium from each well was assayed for HA activity to determine the presence or absence of virus infection. The titre expressed as 50% egg-infective dose/0.1 mL (EID_{50}/0.1 mL) or 50% tissue culture infective dose/0.1 mL ($TCID_{50}$/0.1 mL) was calculated by the method of Reed-Muench (Anon 2000).

2.2.7 Intravenous pathogenicity index (IVPI) test

The IVPI test was performed in an isolation animal house room at the Murdoch University campus by injecting virus with an HA titer > 2^4 intravenously into10 chickens as described in the OIE Manual (OIE 2008). A virus with an IVPI of greater than 1.2 or higher was considered a HPAI. Briefly, the infectious virus (grown in SPF embryonated eggs) with a HA titre 1/512 (2^9, $EID_{50}10^{6.5}$/0.1 mL) was diluted 1/10 in sterile 0.9% NaCl. Aliquots of 0.1 mL of the diluted virus were injected intravenously into ten 6-week-old SAN chickens (Altona Hatchery Pty. Ltd.). Birds were examined for signs of illness at 24-hr intervals for 10 days and scored as follows: 0 if normal, 1 if sick, 2 if severely sick, or 3 if dead. The IVPI score is taken as the average score per bird per observation over the 10-day period, namely the total of all individual chicken scores divided by the total number of observations.

Oropharyngeal and cloacal swabs (OS and CS) were taken on days 1, 3, 5 and 7 and were placed into 1mL of Viral Transport Medium (VTM) (0.1% (w/v) yeast extract (Difco), 0.5% (w/v) lactalbumin hydrolysate, 1x HBSS (Invitrogen), 4000 U/mL penicillin, 10 mg/mL streptomycin, 0.1mg/mL fungizone, 0.035% (w/v) $NaHCO_3$, 0.03% (w/v) glutamic acid, 0.01% (w/v) glutamine, 0.01% (w/v) methionine, 0.015% (w/v) arginine, 0.0001% (w/v) biotin, 0.0001% (w/v) folic acid) provided by DAFWA. These were frozen at -70°C until tested. The birds were bled for the last time and then euthanased by cervical dislocation 10 days post challenge.

2.2.8 Virus challenge

Six 8-week-old SAN chickens were subjected to virus challenge in an isolation animal house room at the Murdoch University campus. Each chicken received 0.5 mL H6N2 virus ($10^{6.5}$ EID_{50} /0.1 mL) by nasal instillation (0.1 mL), eyedrop (0.1 mL) and oral route (0.3 mL). Following challenge all chickens were observed daily. In addition to collecting OS, CS and blood as above, kidney was also taken after the birds were euthanased and a 20% (w/v) homogenate was prepared in VTM for virus isolation.

2.2.9 Virus isolation

Virus isolation was based on standard procedures (OIE 2008; WHO 2002). Each swab was thawed at room temperature, vigorously mixed for 30 sec with a vortex mixer, held at room temperature for 30 to 60 min followed by a clarifying centrifugation at 900 g for 10 min at 4°C. Aliquots of 0.2 mL supernatant fluid from each swab were injected into the allantoic sac of two to four 9 to 11-day-old SAN chicken embryonated eggs. After incubation at 37°C for 6–7 days with periodic candling to remove and store sick or dead

embryos (4°C), harvested allantioc fluids were tested individually for HA activity. The allantoic fluids that gave a negative reaction were re-passaged in chicken embryonated eggs to confirm that a low titre of virus was not present. HA positive eggs were confirmed as H6N2 virus by HI testing as described above (OIE 2008). The frequency of virus shedding was recorded as the number of days, on which positive CS or OS were obtained, divided by the total number of swabs collected post challenge for each group.

2.2.10 Amplification of the H6N2 virus genes of interest

2.2.10.1 Primer design

The 102 AIV H6 sequences from GenBank were aligned using Bioedit (version 5.0.9.1) (Hall 1999). The consensus sequences were used to design the primers for amplification of HA, NP and NA genes of A/coot/WA/2727/79 (H6N2). Recognition sites for restriction endonuclease were added to the 5' ends of the primers as outline in bold in Table 2.1. The feasibility of the designed primers was tested using Amplify 1.2. The primers were synthesized by GeneWorks Pty Ltd (SA, Australia).

Table 2.1 Primers used for amplification and sequencing of A/coot/WA/2727/79 (H6N2) in different vectors.

Primer	Sequence (5'to 3')*	Genome	Nt position**
HAF	aa**CTGCAG**CAAAAGCAGGGGAAAATG***	HA gene	3-20
HAR	cgc**GGATCC**TTTCTAATTATATACATATYYTGC	HA gene	1702-1725
NPF	tgc**TCTAGA**AGCAAAAGCAGGGTAKAT	NP gene	1-18
NPR	cgc**GGATCC**AGTAGAAACAAGGGTATTTTTC	NP gene	1543-1565
NAF	tgc**TCTAGA**AGCAAAAGCAGGAGTBNAAA	NA gene	1-15
NAR	cgc**GGATCC**AGTAGAAACAAGGAGTTTTT	NA gene	1400-1509
M13F	GTTTTCCCAGTCACGAG	pGEM-T vector	2956-3012
M13R	CAGGAAACAGCTATGAC	pGEM-T vector	176-197
VRF	TAGTCTGAGCAGTACTCGTTG	VR1012	1772-1792
VRR	CAATGCGATGCAATTTCCTC	VR1012	2031-2050

* Degenerate nucleotide code Y refers to C or T, K refers to G or T, and N refers to A, C, G or T.
** The nucleotide (nt) numbering system was adopted according to GenBank accession No. CY004515 (HA), CY004518 (NP) and CY004517(NA) (Influenza A virus (A/coot/ALB/134/1987(H6N2)).
*** Restriction endonuclease sequence is in bold. Protective nucleotide bases in lower case were added before recognition sites for restriction endonuclease in order to obtain accurate and efficient digestion.

2.2.10.2 RNA extraction

Viral RNA was extracted using an RNeasy Mini Kit (Qiagen,VIC, Australia) according to the manufacturer's instructions. In brief, H6N2 AIV infected allantoic fluid was centrifuged at 2,000 g at 4°C for 30 min. The supernatant was then centrifuged at 180,000 g at 4°C for 3 hr. The pellet was subsequently dissolved in a 1/10 volume of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0) before being mixed with an equal volume of RLT Buffer (plus β-mercaptoethanol). Following centrifugation at 13,000 g for 1 min, the supernatant was transferred to a new microcentrifuge tube. An equal volume of 70% ethanol was added to the supernatant followed by transfer to an RNeasy spin column placed in a 2 mL collection tube. The column was subsequently centrifuged for 15 sec at 10,000 g. After discarding the flow-through, 700 µL Buffer RW1 was added to the RNeasy column followed by centrifugation for 15 sec at 10,000 g and the flow-through discarded. Following this, 500 µL Buffer RPE was added onto the RNeasy

column and subsequently centrifuged for 15 sec at 10,000 g and the flow-through discarded. After the RNeasy column was washed again with 500 µL Buffer RPE, it was centrifuged for 2 min at 10,000 g to remove any residual wash buffer. The RNeasy column was then transferred to a new 1.5 mL collection tube and 50 µL of RNase-free water was added onto the RNeasy silica-gel membrane and allowed to sit at room temperature for 10 min. RNA was eluted by centrifugation for 1 min at 10,000 g . The RNA concentration was measured using a nanodrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA), and then stored at -70 °C until use.

2.2.10.3 Reverse Transcription (RT)

cDNA was synthesized using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) following the protocol recommended by the manufacturer. Briefly, in a total volume of 50 µL, 25 µL RNA was mixed with 13.5 µL nuclease-free dH_2O, 5.0 µL of 10x RT buffer, 2.0 µL of 25x dNTP Mix (100 mM), 2 µL of 20 µM forward primer and 2.5 µL (50 U/µL) MultiScribe^{TM} Reverse Transcriptase. Following gentle mixing, RT was performed in a thermal cycler using the following cycle protocol: 25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, and 4°C hold. The reaction was kept at 4°C for short term storage or -20°C for long term storage.

2.2.10.4 Gradient PCR for determining optimal annealing temperature

Gene fragments of interest were amplified by gradient PCR using a PCR Thermal Cycler (Eppendorf Mastercycler Gradient PCR Thermal Cycler, NY, USA). Briefly, in a 50 µL volume, 29.75 µL dH_2O was mixed with 10 µL of 5 x Green GoTaq® Flexi Buffer, 5 µL of 5 mM $MgCl_2$, 1 µL of dNTP mix (10 mM each), 1 µL of 30 µM forward primer, 1 µL

of 30 μM reverse primer, 2 μL cDNA and 0.25 μL GoTaq® DNA polymerase (5 U/μL) . After aliquoting 10 μL per PCR tube, amplification was performed in a PCR thermal cycler using the following parameters: one cycle of 95°C for 2 min; 40 cycles of 95°C for 40 sec, different annealing temperatures for 30 sec, 72°C for 4 min, and a final extension step of 72°C for 7-10 min.

2.2.10.5 PCR using proof-reading polymerase

PCR reactions were performed in a 50 μL volume containing 37.25 μL dH_2O, 5 μL Pfu DNA polymerase 10 x buffer with $MgSO_4$, 1 μL of dNTP mix (10 mM each), 1 μL of 30 μM forward primer, 1 μL of 30 μM reverse primer, 4 μL cDNA template, 0.5 μL of Pfu DNA polymerase (3 U/μL, Promega Corp., Sydney, Australia), 0.25 μL GoTaq® DNA polymerase (5 U/μL, Promega). Amplification was conducted under the following thermal cycling conditions: one cycle of 95°C for 2 min; 40 cycles of 95°C for 40 sec, 52.5°C for 30 sec, 72°C for 4 min, and a final extension step of 72°C for 7-10 min.

2.2.11 Agarose gel electrophoresis

PCR products were visualized in a 1-1.2% agarose gel with ethidium bromide (EB) added to give a final concentration of 0.5 μg/ mL. Prior to loading, samples were mixed with 6x loading buffer (Promega). Size markers (100 bp DNA ladder plus (Fermentas, QLD, Austrlia), or 1 kb DNA ladders (Promega) were added to separate lanes. Electrophoresis was conducted in TAE buffer (242 g Tris base, 57.1 mL glacial acetic acid, 100 mL of 0.5 M EDTA (pH 8.0) in 1000 mL dH_2O) at a constant voltage of 60-120 kV for 40 min to 2 hr depending on the resolution required. Gels were checked with an ultraviolet transilluminator to visualize DNA bands.

2.2.12 Estimation of nucleic acid concentration

The concentration of DNA was estimated photometrically by measuring absorption of the sample at 260 nm using a NanoDrop ND-1000 Spectrophotometer. The purity of nucleic acid was determined by the OD_{260}/OD_{280} ratio. Pure DNA has an OD_{260}/OD_{280} ratio of ~1.8 whereas pure RNA has an OD_{260}/OD_{280} ratio of ~2.0.

2.2.13 Recovery of DNA from agarose gels

Following agarose gel electrophoresis, DNA bands of interest were recovered using MiniElute gel extraction kit (Qiagen) according to the manufacturer's instructions. Briefly, following electrophoresis at 70 V for 60- 90min, the bands were cut using a clean scalpel blade under long UV light/ illuminator if EB was used or under Safe Imager blue-light transilluminator if the SYBR green I dye (Invitrogen) was used.

Following excision 3 times the gel volume of buffer QG was added and incubated at 50°C for 10 min. Following the addition of 1 gel volume of isopropanol, the mixture was loaded onto a QIAquick spin column in a 2 mL collection tube provided. After centrifugation at 13,000 g for 60 sec, the flow-through was discarded and 0.5 mL QG was added followed by the same centrifugation as before. The column was washed twice with 750 µL buffer PE by centrifugation (13,000 g for 60 sec and then 2min). The column was transferred to a 1.5 mL tube before 10-30 µL of dH_2O was added and incubated at room temperature for 10 min. The DNA was eluted from the membrane by centrifugation at 13,000 g for 60 sec.

2.2.14 Cloning of PCR products

2.2.14.1 Ligation reactions

The pGEM®-T Easy vector (Promega) was used to clone PCR products and served as a holding vector for PCR products. Molar ratios of gene of interest versus vector of approximately 3:1 to 5: 1 were used in the ligation reactions. The ligation followed the protocol of the pGEM-T Easy vector kit. The reaction mixture containing 5 µL of 2x rapid ligation buffer, 3 µL of DNA fragment, 1 µL T4 DNA ligase (3 U/µL, Promega), 1 µL pGEM-T Easy vector (50 ng/µL) was gently mixed and incubated at 4°C overnight.

2.2.14.2 Transformation

Transformation was conducted using JM109 competent *Escherichia coli* (*E. coli*) cells (Promega). Briefly, the cells were thawed on ice after which a 5 µL of ligation reaction was added to 50 µL of competent cells followed by gentle mixing and incubating on ice for 15-20 min. The reaction was subsequently placed at 42°C for 45-50 sec and immediately put back on ice for 2 min. Following the addition of 900 µL Luria-Bertani (LB) media (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, adjust the pH to 7.0 with 5N NaOH and the volume to 1 L with dH_2O) the mixture was incubated at 37°C with shaking (225 rpm) for 1 hr. During this period, 1.2% agar (1.2% bacto agar added to LB media) plates containing100 µg/mL ampicillin, 100 µL of 100 mM IPTG and 20 µL of 50 mg/mL X-gal were prepared for selective blue-white screening. Following incubation, 100 µL and 200 µL culture were spread onto plates, followed by incubation at 37°C overnight. White colonies were subsequently chosen for PCR screening.

2.2.14.3 PCR for screening of recombinant plasmids

A single white colony was randomly picked and re-streaked on a new plate supplemented with 100 µg/mL ampicillin, 0.5 mM IPTG and 80 µg/mL X-gal, followed by incubation

at 37°C overnight. Replicate colonies were then picked into 20 μL dH_2O. Following boiling for 10 min, the mixture was used as a template for PCR.

PCR was conducted with a volume of 20 μL PCR reaction containing 4 μL of 5 x PCR buffer, 2 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP, 0.25 μL of 30 μM M13F, 0.25 μL of 30 μM M13R, 0.1 μL Taq polymerase(5 U/μL), 5 μL template DNA, and 7.4 μL dH_2O. The thermal cycle conditions were as follows: 95 °C for 4 min; 94 °C for 40 sec, 56 °C for 30 sec, 72 °C for 90 sec, 35 cycles; 72 °C for 7 min. After electrophoresis, positive clones were identified by visualization of bands of expected size. Sequencing of these positive clones was used to further confirm their identity.

2.2.15 Plasmid DNA purification

A QIAprep®Spin Miniprep kit (Qiagen) was used for purifying plasmid DNA. In brief, one colony was inoculated into 3-5 mL LB with100 μg/mL ampicillin followed by incubation at 37°C overnight. The culture was centrifuged at 13,000 g for 1 min and the resulting pellet dissolved in 250 μL of Buffer P1. The cells were lysed with 250 μL Buffer P2, followed by addition of 350 μL Buffer N3 until a precipitate formed. After the mixture was centrifuged at 13,000 g for 10 min at room temperature, 800 μL of supernatant containing plasmid DNA was transferred to a spin column. After centrifugation at 13,000 g for 40-60 sec, the flow-through was discarded. The column was washed once with 0.5 mL of Buffer PB and twice with 750 μL of buffer PE by centrifugation at 13,000g for 40-60 sec. Following centrifugation at 13,000 g for 2 min to remove residue buffer PE, the column was placed into in a 1.5 mL tube. 50 μL dH_2O was added and left for 10 min at room temperature. The DNA was eluted by centrifugation at 13,000 g for 60 sec before being quantified and finally stored at -20 °C.

2.2.16 Sequencing

Nucleotide sequencing was conducted using ABI PRISM^{TM} Big Dye Terminator Version 3.1 ready reaction kit with AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Conn., USA) in an ABI 377 automatic DNA sequencer (Applied Biosystems, USA). In brief, 10 µL reactions containing 1 µL of 5X sequencing buffer, 1 µL of 3.2 pmoles/µL M13F or M13R primer, 5 µL template (80 ng/µL plasmid DNA), 2 µL of Dye terminator mix, 1 µL dH_2O were subjected to thermal cycling conditions of 96 °C for 2 min; followed by 25 cycles of 96°C for 10 sec, 56°C for 5 sec and 60°C for 4 min, with a 14°C final hold. DNA was precipitated by adding 25 µL of 100% ethanol, 1 µL of 125 mM EDTA and 1 µL of 3M NaOAc (pH 4.8) on ice for 20 min followed by centrifugation at 14,000 g for 30 min at room temperature. Pellets were rinsed with 125 µL of 70% ethanol and dried for 5-10 min at room temperature. The pellet was stored at -20 °C until sequencing by qualified SABC personnel. Sequence data was analysed using SeqEd^{TM} version 1.0.3 (Applied Biosystem Inc.).

2.2.17 Sequence analysis and construction of phylogenetic tree

The HA amino acid sequence of A/coot/WA/2727/79 (H6N2) was deduced by EMBOSS Transeq (http://www.ebi.ac.uk/Tools/emboss/transeq/) and then aligned with selected LPAI or HPAI isolates by Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to analyse the cleavage region sequence.

Phylogenetic analysis of the nucleotide sequences obtained from this study and from the GenBank database was performed using the Molecular Evolution Genetics Analysis program (MEGA, version 4.0)(Tamura *et al.* 2007). Reference viruses were selected from

representative isolates of H1-H16 and N1-N9 subtype as well as some Australian isolates, for which corresponding complete sequences of three genes were available from GenBank. The estimated evolutionary distances between DNA sequences were displayed by Maximum Composite Likelihood model with the neighbor-joining method with 1,000 bootstrap replications.

2.2.18 Data analysis

All statistical analysis of HI antibody titres was conducted as geometric mean titres (GMT). Statistical analyses of experimental data and controls were conducted by using the paired-sample T-test, and one way analysis of variance (ANOVA) (Mann-Whitney Test was used as homogeneity of variance testing showed statistical significance) with SPSS statistical software version 15 as well as Chi-square (Fisher's exact test was used when the sample number was less than 5) using Statistix. Statistical significance was defined at the level of $p<0.05$.

2.3 Results

2.3.1 Virus growth in cell cultures

Monolayers of MDCK cells in the 6-well plate inoculated with 100 μL of 1/10 dilution of allantoic fluid containing A/coot/WA/2727/79 (H6N2) showed morphological changes consistent with virus induced cytopathic effect (CPE), including rounding and shrinkage of cells, plaque formation and finally detachment of cells from the surface of culture plate. In contrast, uninfected cells showed no sign of cell damage (Figure 2.1 and 2.2).

Interestingly, this H6N2 LPAI virus grew in MDCK cells in the absence of exogenous trypsin. The virus titre in the supernatant of infected cells was $10^{6.5}$ $TCID_{50}$/0.1mL (HA

titre 2^8) in the presence of TPCK and $10^{5.5}$/0.1mL $TCID_{50}$ (HA titre 2^6) in the absence of TPCK.

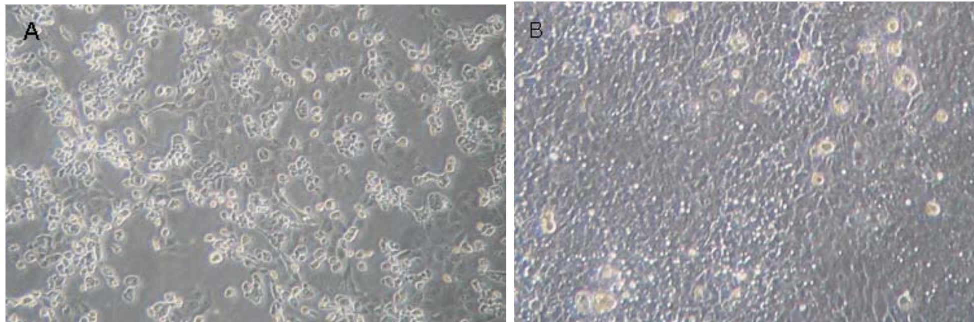

Figure 2.1 Morphological changes postinoculation of A/coot/WA/2727/79 virus in MDCK.
A, MDCK with CPE; B, uninfected control cells (x10).

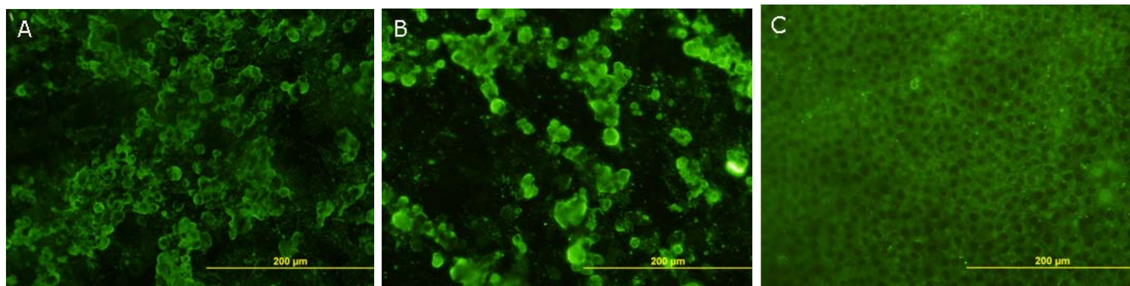

Figure 2.2 Immunofluorescent staining of virus infected MDCK cells.
A, cultures with TPCK trypsin; B, cultures without TPCK trypsin; C, uninfected control cells, (x20).

2.3.2 Intravenous pathogenicity index

After IV inoculation of the H6N2 virus the unvaccinated challenged chickens consumed less feed during the first 7 days but their appetite returned to normal after that. Correspondingly, these birds looked smaller compared to the vaccinated and naïve control groups, however, body weight measurements were not conducted. This suggested that virus intravenous inoculation resulted in mild growth suppression. No birds showed any other clinical signs during a 10-day observation period. One out of ten birds died on

day 6 following IV injection. Thus, the IVPI score of this virus was 0.15. According to the criteria of the World Organisation for Animal Health (OIE 2008), the isolate was classified as a LPAI virus.

2.3.3 Comparison of virus shedding by different challenge routes

Following IV challenge, the remaining nine birds showed a HI GMT of 2^7 (titres ranging from 2^5 to 2^8) by 10 days post inoculation whereas six birds showed HI GMT of 2^6 (titres ranging from 2^4 to 2^9) after combined oral and oculonasal challenge (Figure 2.3). Moreover, there was no significant difference in the GMT (ANOVA, P=0.219) post-challenge between IV route, and combined oral and oculonasal routes, however, there was a significant difference (ANOVA, P=0.000) in the GMT between prior and post-challenge in both IV challenge group and the combined oral and oculonasal groups, indicating that this virus can replicate in chickens along with an absence of clinical signs.

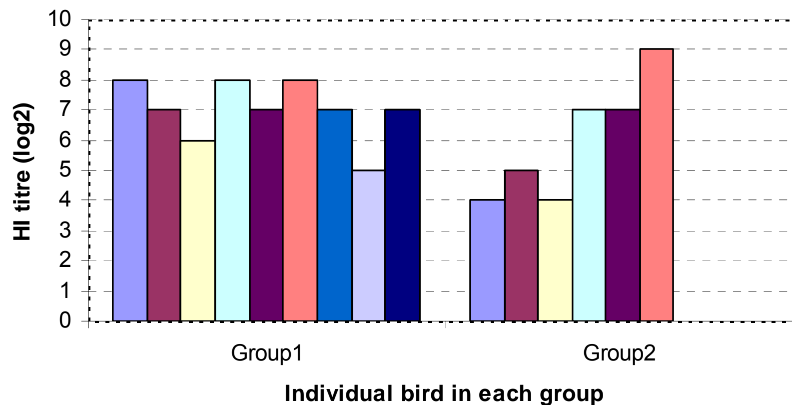

Figure 2.3 HI titre after virus challenge via intravenous route (group1) and combined oral and oculonasal routes (group2).

Table 2.2 Virus shedding in swabs post challenge via intravenous as well as combined oral and oculonasal route.

	Intravenous challenge																				Oral and oculonasal challenge											
	241		242		243*		244		245		266		267		268		269		270		231		232		233		234		235		236	
	O**	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C
D1	+***	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
D3	+	–	–	–	+	–	–	–	+	–	–	–	+	–	–	+	–	–	+	–	+	–	+	+	+	–	+	–	–	–	+	–
D5	–	–	–	–	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–	–	+	–
D7	–	–	–	–	–	+	–	+	–	–	+	–	–	+	–	–	–	+	–	–	–	–	+	–	–	–	–	+	–	–	+	–

* The bird died on day 6 post challenge. Swabs on day 7 referred to that on day 6. ** O represents oropharyngeal swabs and C represents cloacal swabs.
*** + positive and – negative for virus isolation.

As shown in Table 2.2, the virus shedding rate post-challenge in OS was 25% (10/40) and 70.8% (17/24) whereas the rate in CS was 20% (8/40) and 12.5% (3/24) respectively for the IV route, and the oral/oculonasal routes. There was a highly significant difference (Chi-square, P=0.0003) in virus shedding via OS and no significant difference (Fisher exact tests, P=0.51) in virus shedding via CS between the IV route and the oral/ oculonasal routes. Moreover, no virus was detected in the kidneys 10 days post-challenge.

2.3.4 Cloning and sequencing of avian influenza virus gene

The viral HA, NP and NA genes of the H6N2 virus were amplified from infective allantoic fluid by RT and PCR (Figure 2.4), showing expected sizes, 1.7 kb for HA, 1.5 kb for NA and 1.6 kb for NP. These three genes were cloned into pGEM-T easy vector to generate pT-HA, pT-NP and pT-NA, and the cloned genes were respectively amplified by M13 and virus primers as shown in Figure 2.4.

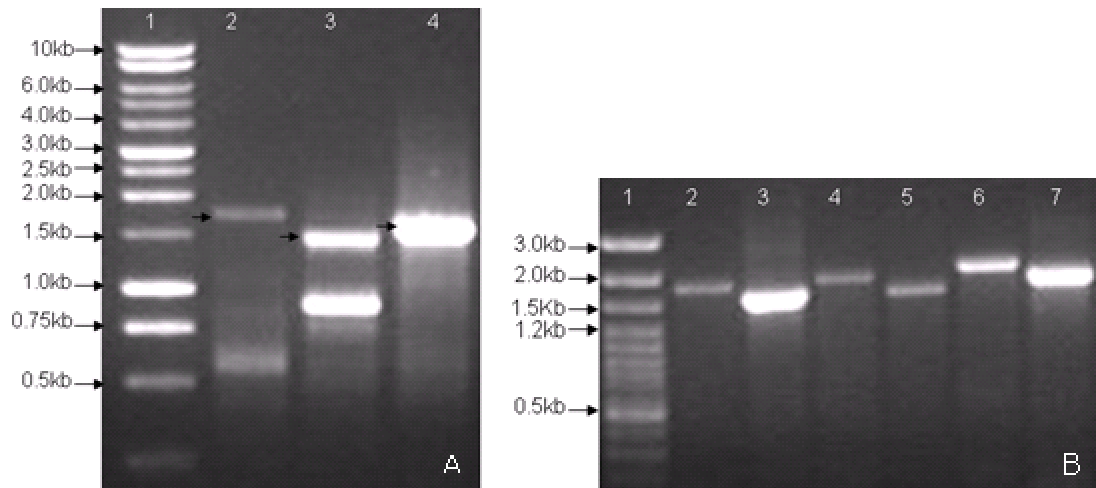

Figure 2.4 A RT-PCR amplification of HA, NP and NA gene of the H6N2 virus. Lane 1, 1kb marker (Promega); lane 2, HA; lane 3, NA; lane 4, NP fragment (arrow indicates specific fragment). **B PCR amplification of HA, NP and NA genes in pGEMT-easy vector using M13 and virus primers.** Lane 1, 100bp marker (Fermentas); lane 2, NA-M13 primers; lane 3, NA-virus primers; lane 4, NP-M13 primers; lane 5, NP-virus primers; lane 6, HA-M13 primers; lane 7, HA-virus primers.

Three segments of genomes of A/coot/WA/2727/79 (H6N2) have been sequenced and are shown in Appendix 2.1. The complete open reading frame (ORF) of A/coot/WA/2727/79 (H6N2) virus HA gene was determined to be 1701 nucleotides long, capable of encoding a protein of 567 amino acids. The deduced amino acid sequence showed no multiple basic amino acid motif in the cleavage site of the HA, which is characteristic of HPAI viruses (Figure 2.5). In comparison with the presence of leucine (L) and serine (S) at residues 226 and 228 considered as conserved amino acid sites for receptor binding for human influenza viruses, this isolate had respectively glutamine (Q) and glycine (G) residues (Figure 2.6). The HA sequence was 1723 bp long. The HA ORF sequence is the same as that in GenBank accession number (CY028243) (1709 bp).

```
AF474035         GALRTNKTFQNVSPLWIGKCPKYVKSESLRLATGLRNVPQIA----TRGLFGAIAGFIEG 356
D90303           GVLRTNKTFQNVSPLWIGECPKYVKSESLRLATGLRNVPQIE----TRGLFGAIAGFIEG 356
CY005691         GALRTNKTFQNVSPLWIGECPKYVKSDSLRLATGLRNVPQAE----TRGLFGAIAGFIEG 356
A/Coot/2727/79   GALRTNKTFQNVSPLWIGECPKYVKSDSLRLATGLRNVPQAE----TRGLFGAIAGFIEG 356
CY004515         GAINTNKTFQNISPVWIGECPKYVKSKSLKLATGLRNVPQAQ----TRGLFGAIAGFIEG 356
AF028709         GAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNTPQRERRRKKRGLFGAIAGFIEG 358
AY577314         GAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEG 358
AY497096         GAINSSMPFHNVHPFTIGECPKYVKSKKLVLATGLRNVPQRE----TRGLFGAI------ 332
AY497093         GAINSSLPFHNVHPFTIGECPRYVKSKKLVLATGIRNVPQRE----TRGLFGAI------ 332
```

Figure 2.5 Alignment of the HA cleavage region among A/coot/WA/2727/79 and reference isolates. AF474035(A/chicken/ California/6643/2001(H6N2)), D90303(A/shearwater/Australia/1/1972(H6N5)), CY005691 (A/black duck/AUS/4045/1980(H6N5)), CY004515 (A/coot/ALB/134/1987(H6N2)), AF028709 (A/Hong Kong/156/97(H5N1)), AY577314 (A/Thailand/3(SP-83)/2004(H5N1)), AY497096 (A/chicken /Mexico/232/94 (H5N2)), AY497093 (A/chicken/El Salvador/102711-1/01 (H5N2)).

```
EF626617_A/Memphis/102/72(H3N2)        IWGVHHPSTDQEQTSLYVQASGRVTVSTKRSQQTIIPNIGSRPWVRGQSS 228
M54895_A/Udorn/307/72(H3N2)            IWGVHHPSTDQEQTSLYVQASGRVTVSTKRSQQTIIPNIGSRPWVRGLSS 243
EF626609_A/Victoria/3/75(H3N2)         IWGVHHPSTDKEQTDLYVQASGKVTVSTKRNQQTVIPNVGSRPWVRGLSS 228
EF626610_A/Bangkok/1/79(H3N2)          IWGVHHPSTDKEQTNLYVRASGRVTVSTKRSQQTIIPNIGSRPWVRGLSS 228
EF614248_A/Aichi/2/1968_H3N2           IWGVHHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIESRPWVRGLSS 243
M24720_A/equine/Tokyo/71(H3N8)         VWGIHHPSTNNEQTKLYVQASGRVTVSTKRSQQTILPNIGLRPWVRGQSG 243
CY032953_A/eq/Johannesburg/86(H3N8)    IWGIHHPSSNNEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPWVRGQSG 243
A/coot/WA/2727/79(H6N2)                FWGVHHPPDTNEQNTLYGSGDRYVRMGTESMNFAKSPEIAARPAVNGQRG 241
```

Figure 2.6 Comparison of amino acid at position 226 and 228 of HA.

The NP gene of A/coot/WA/2727/79 (H6N2) was 1565 bp whereas the NCBI sequence (accession No. CY028246) was 1530 bp. The ORF of NP gene was determined to be 1494 nucleotides long, capable of encoding a protein of 498 amino acids. The NP sequence in the ORF region is the same as that in NCBI.

The NA gene of A/coot/WA/2727/79 (H6N2) was 1468 bp whereas the NCBI sequence (accession No. CY028245) was 1434 bp. The ORF of NA gene was determined to be 1407 nucleotides long, capable of encoding a protein of 469 amino acids. There were six base differences between our sequence and the NCBI sequence, which resulted in five amino acid differences (Figure 2.7).

```
gi|162373910|gb|CY028245.1|      -------------AGTGAAAATGAATCCAAATCAGAAGATAATAACAATT 37
NA_29Mar07_                      AGCAAAAGCAGGAGTTGAAAATGAATCCAAATCAGAAGACAACAACAATT 50
                                                ************************ ** *******

gi|162373910|gb|CY028245.1|      GGCTCTGTCTCTCTAACCATCGCAACAATATGCTTTCTCATGCAGATTGC 87
NA_29Mar07_                      GGCTCTGTCTCTCCAACCATCGCAACAACATGCTTTCTCATGCAGATTGC 100
                                 ************* ************** *********************
```

Figure 2.7 Comparison of NA gene sequence between this study and the GenBank.

2.3.5 Phylogenetic analysis

Phylogenetic trees based on HA, NA and NP genes of the A/coot/WA/2727/79 (H6N2) virus are shown in Figure 2.8. Phylogenetic analysis of the HA gene demonstrated that A/coot/WA/2727/79 (H6N2) shared the highest sequence identity with influenza A virus (A/black duck/AUS/4045/1980 (H6N5)) (Genbank accession CY005691). There were wide differences between this H6N2 virus and the other Australian isolates of different subtypes prevalent in Australian wild birds at that time in the database, including A/red-necked stint/AUS/4500/1980 (H3N8) (CY005705), A/gray teal/AUS/3/ 1979 (H4N6)

(CY005682), A/red-necked stint/AUS/5745/1981 (H12N9) (CY005711), A/wedge-tailed shearwater/Western Australia/2576/1979 (H15N9) (CY005408). This isolate was also closely related to other H6 avian influenza viruses, especially isolates from Alberta.

From the phylogenetic tree the H6 avian influenza virus group appeared to be closer in virus evolution to H1, H2 and H5 virus groups than to H3, H4, H7, H8,H9, H11,H12, H13, H15 (there was insufficient data available in Genbank to extend these relationships to H10, H14 and H16 viruses).

Four groups can be assigned from the phylogenetic analysis of NA genes: (i) N1, N4; (ii) N5, N8; (iii) N3, N6, N7, N9; and (iv) N2, which was consistent with the previous grouping of NA genes (Harley *et al.* 1989). The phylogenetic analysis of NA genes clearly supported the subtyping of the A/coot/WA/2727/79 virus as H6N2. All the N2 subtype viruses examined showed close phylogenetic relationships to each other including North American and Asian isolates. N2 groups showed substantial phylogenetic differences from other N subgroups. In an evolutionary sense the N1 subtype appeared to be closer to N4 subtype, N5 appeared closer to N8 subtype and N6 appeared closer to N9 subtype.

The phylogenetic analysis of the NP genes demonstrated that A/coot/WA/2727/79 (H6N2) shared the highest sequence identity with Influenza A virus A/black duck/AUS/4045/1980 (H6N5) (Genbank accession CY005694). It also had a closer relationship with other Australian isolates than those from Asia, North America or Europe.

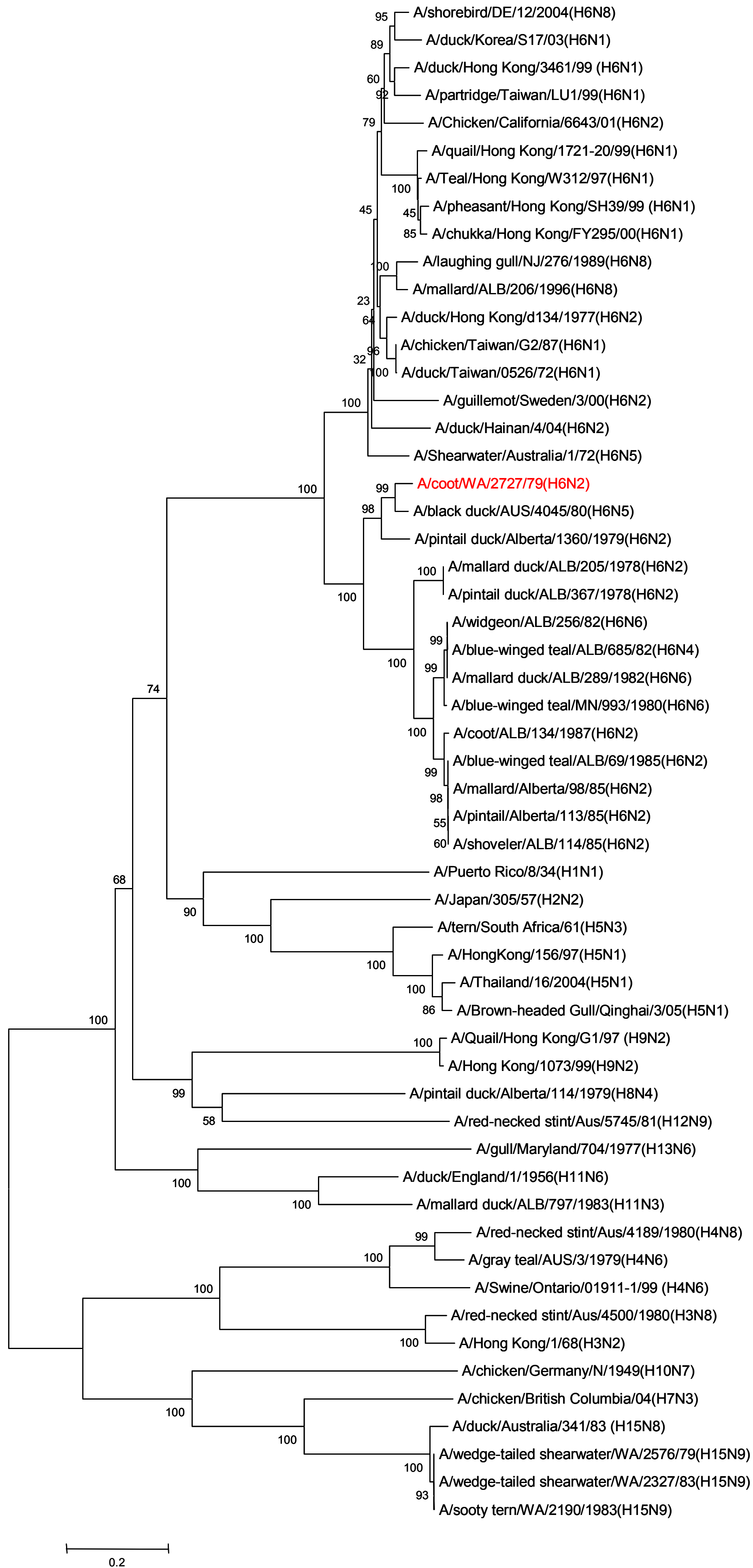

Figure 2.8a Genetic relationship between representative strains for HA gene.

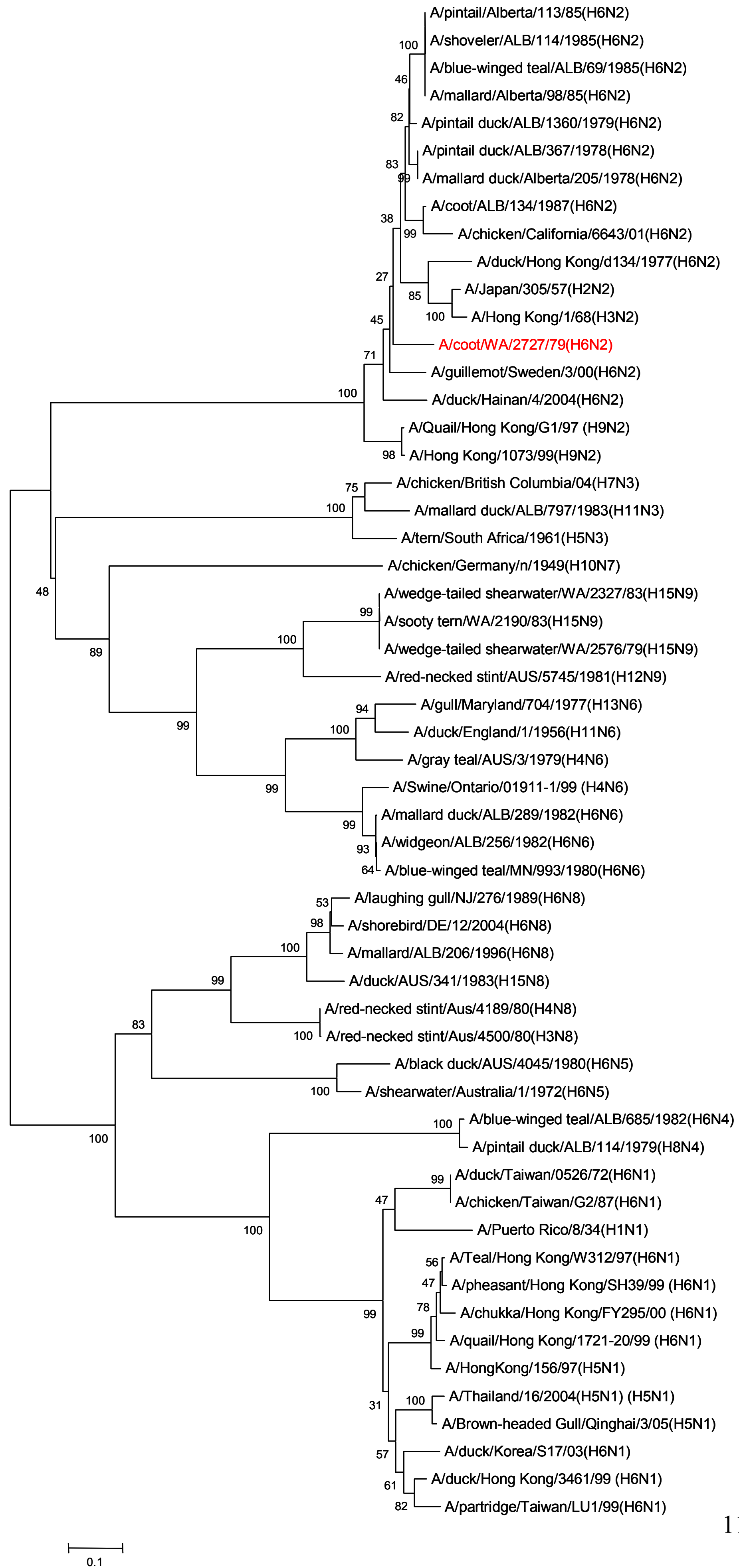

Figure 2.8b Genetic relationship between representative strains for NA gene.

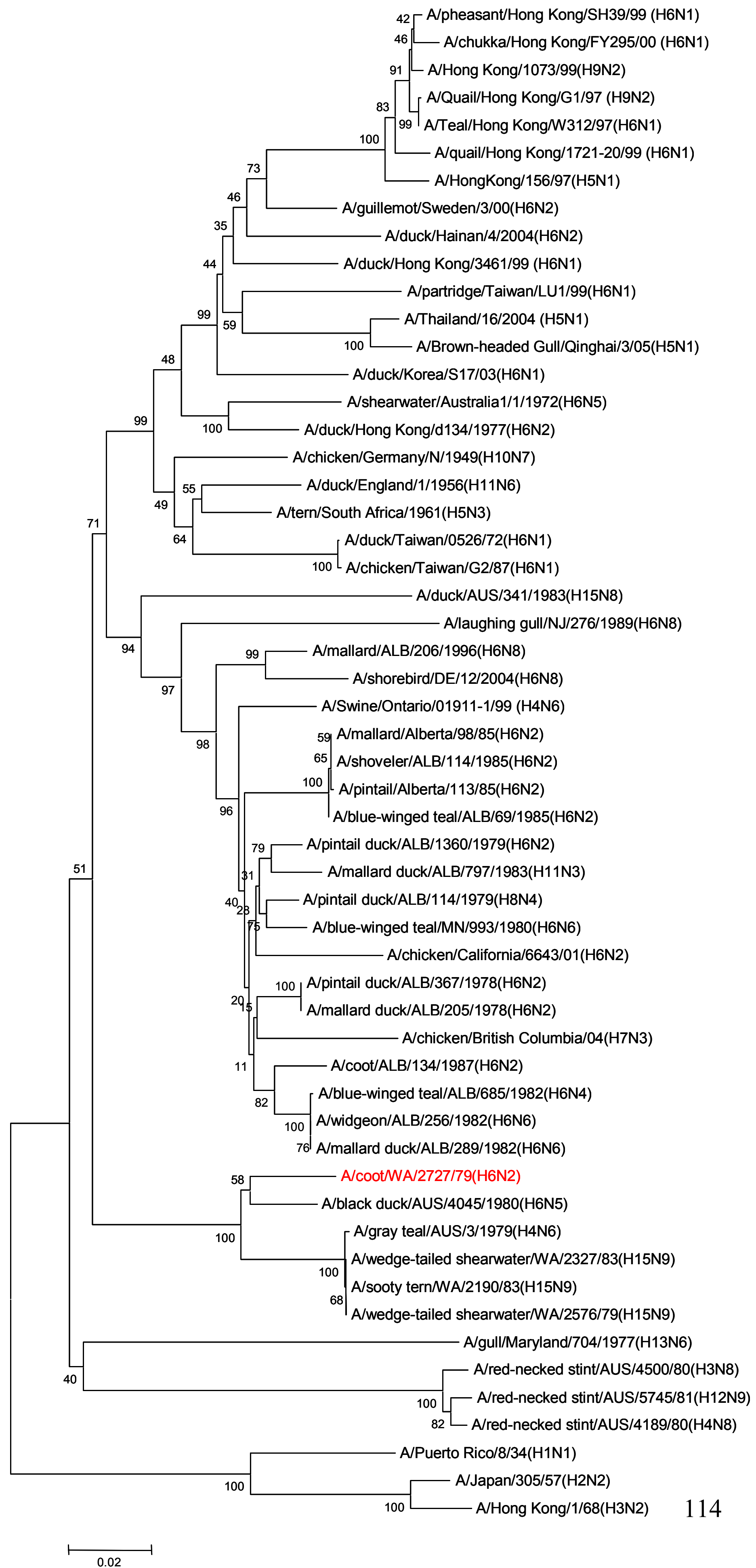

Figure 2.8c Genetic relationship between representative strains for NP gene.

2.4 Discussion

This study describes the biological and genetic properties of A/coot/WA/2727/79 (H6N2) isolated from an apparently healthy coot in 1979. This virus was classified as LPAI based on an IVPI score of 0.15 and a motif of ^{321}PQAETRG^{328} at the HA cleavage site, which did not present the multiple basic acid motif found in HPAI viruses. Moreover, this isolate is able to grow on commonly used MDCK cells in the absence of exogenous TPCK trypsin.

Accumulated data has indicated that new pandemic human influenza viruses arising every 10-15 years are derived from AIVs isolated from lower mammals or birds either by direct transmission or by genetic recombination between human and animal viruses (Alexander and Brown 2000; Downie *et al.* 1977; Hobson 1973). As AIVs of wild birds may serve as progenitors of human pandemic influenza viruses, investigation and analysis of these viruses can provide useful information on the spectrum of AIVs existing in wild bird populations around the world. This may assist in identifying viruses with the potential to evolve into human pandemic influenza viruses. Moreover, identifying and assessing the biological properties and potential pathogenicity of waterfowl-origin type A influenza viruses in chickens could help identify viruses that present an increased threat to domestic poultry.

The HA glycoprotein for influenza A viruses is responsible for virus attachment to host cells and then fusion between the host cell membrane and the virus membrane (Alexander 2008). This glycoprotein is synthesized as a precursor, HA0, which requires post-translational cleavage by host proteases before it is able to induce membrane fusion and before infectious virus particles can be produced (Rott 1992). In general, the HA0

precursor in LPAI viruses is cleaved only in certain environments where trypsin-like enzymes are present or exogenous trypsin is added for the efficient replication of the virus in cell culture (Lee *et al.* 2008). In the current study the H6N2 virus replicated in MDCK cells without addition of exogenous trypsin, although replication, as measured by $TCID_{50}$ and HA titre in the supernatant, was more efficient in the presence of trypsin. In this regard the H6N2 isolate behaved more like an HPAI virus in cell culture despite being clearly LPAI by the gold standard IVPI tests. The reason for this was not determined in this study. Possible causes could include the presence of residual trypsin during cell passage, however the growth media used included sufficient FCS to inactivate any remaining trypsin. Alternatively, the MDCK cells may have sufficient protease activity that permitted cleavage of the HA0 of this particular H6N2 virus. In an experiment with *in vitro* cultivation of swine influenza virus, HA titers in the presence of trypsin reached a maximum of 1: 2048 (average titer, 1: 870) whereas the titres were 1: 4 to 1: 8 (average titre, 1:2.6) without trypsin (Herman *et al.* 2005). This indicated that some low pathogenic viruses have ability to grow without trypsin.

Some studies showed that all H6 AIVs examined contained the amino acid motif PQIETR↓G at the cleavage site (arrow) between HA1 and HA2 (Chin *et al.* 2002; Webster and Rott 1987). However, the current H6N2 virus and the closely related A/black duck/AUS/4045/1980(H6N5) and A/coot/ALB/134/1987(H6N2) viruses possessed PQAETR↓G (Figure 2.5). Possibly this motif allowed HA0 cleavage by weak cellular proteases for these viruses.

The HA of influenza type A viruses is responsible for binding of the virus to the cell surface of sialic acid (SA) containing oligosaccharide receptors (Connor *et al.* 1994;

Vines *et al.* 1998). The amino acids at the receptor-binding site (RBS) of HA are highly conserved among AIVs. In H2 and H3 influenza virus strains (human viruses), the presence of leucine at position 226 and serine at 228 in the HA was correlated with preferential recognition of SAα2,6Gal, whereas among equine and avian influenza viruses, the presence of glutamine at residue 226 and glycine at residue 228 preferentially recognize SAα2,3Gal (Connor *et al.* 1994)). Moreover, the HA is responsible for host range restriction of influenza A virus. Amino acid residue analysis of this isolate further highlights that this virus has a specificity for avian and inability to bind SAα2,6Gal despite an ability to grow on MDCK cells in the absence of Trypsin.

Waterfowl are considered to be the principal reservoir of Influenza A viruses. Variations in pathogenicity may exist among AIVs isolated from wild birds. Slemons *et al.*(1991) assessed the pathogenicity potential of 29 wild duck-origin influenza A viruses with a range of HA-NA combinations (including H5N1, H5N2, H5N9, H7N8). IVPI indices of all these viruses ranged from 0.0 to 0.49. A total of seven out of 232 chickens died following IV challenge with 29 waterfowl-origin influenza A viruses. All deaths fell within 3-7 days postinoculation. For the IVPI conducted on the H6N2 virus in this study only one bird died on day 6. The OIE criteria for classifying an AIV as HPAI is any virus with an IVPI greater than 1.2 or where six or more out of eight inoculated chickens die (OIE 2008). Thus, the H6N2 virus tested in the present study was classified as an LPAI virus. Growth suppression may be an objective measure of the pathogenicity potential of LPAI (Karunakaran *et al.* 1988). In our experiment, a period of inappetance and apparent growth suppression but no other clinical signs were observed. In view of these observations the H6N2 virus used in these studies was classified as an LPAI virus.

H6 antibody responses appeared in the chickens after administering this virus via either the IV route or the combined oral and oculonasal routes. This further verified previous studies that waterfowl-origin Influenza A viruses are capable of infecting domestic chickens under experimental conditions but without causing clinical illness (Condobery and Slemons 1992; Slemons *et al.* 1991; Slemons and Swayne 1990, 1995). Moreover, virus shedding following virus challenge via different routes presented obvious differences. Relatively even levels of excretion via the cloaca and oropharynx occurred following IV challenge. However, a higher shedding rate from the oropharynix resulted from oral and oculonasal inoculation, which implied a high level and sustained virus growth in the upper respiratory tract.

The NP gene of A/coot/WA/2727/79 (H6N2) was more closely related to other Australian isolates than isolates from Asia, North America or Europe, indicating that these Australian isolates may have common ancestral nucleoprotein genes. The H6 HA and NP phylogenetic trees revealed that this H6N2 virus isolate was closely related to A/black duck/AUS/4045/80(H6N5) and to other AI strains isolated from shearwaters in Australia from 1979 to 1983. This suggested that this genetic type was prevalent in Australian wild birds during that time. The H6 HA and NA phylogenetic trees revealed that this isolate was related to North America lineage viruses (e.g. A/pintail duck/Alberta/1360/1979 (H6N2) and A/coot/ALB/134/1987 (H6N2)) and also to Eurasian lineage viruses (e.g. A/duck/Hong Kong/d134/1977 (H6N2), A/guillemot/Sweden/3/00 (H6N2)), indicating that interaction may have been occurring among migratory wild birds between two geographic regions at that period. The isolation

of a similar virus from pelagic birds in Australia showed how widely these AIVs are spread around the world.

Since this H6N2 strain itself was not further purified before cloning the genes of interest, the possibility that the virus stock was polyclonal was not eliminated. It is possible that minor differences in sequences could have resulted from the clones chosen for sequencing. The sequences for the genes from this virus lodged in Genbank resulted from sequencing after different passage histories in embryonated chicken eggs in different laboratories. All these factors may have contributed to the six nucleotide differences in the NA gene between the sequence obtained in this study and the GenBank sequence for this virus.

Chapter 3

Development and evaluation of haemagglutinin- or nucleoprotein-expressing DNA vaccines in a VR1012 expression vector against avian influenza H6N2 virus in chickens

3.1 Introduction

DNA vaccines offer some distinct advantages over traditional protein-based vaccines due to their safety, effectiveness, stability and lack of reliance on egg or cell culture-based production (Doria-Rose and Haigwood 2003; Forde 2005). DNA vaccines expressing the HA or NP gene of AIVs have provided solid immune protection against influenza virus infections in chickens (Chen *et al.* 2001; Cherbonnel *et al.* 2003; Fynan *et al.* 1993a; Fynan *et al.* 1993b; Jiang *et al.* 2007; Kodihalli *et al.* 1997; Kodihalli *et al.* 2000; Suarez and Schultz-Cherry 2000b). However, immunogenicity of DNA vaccines in chickens has been variable depending on expression vector, insert gene (antigenicity of encoded insert) and the delivery method and route of inoculation (Kodihalli *et al.* 1997; Kodihalli *et al.* 2000; Robinson *et al.* 1993). In addition, the experimental procedures that are normally used in these studies, which include either administering large doses of plasmid DNA multiple times (Suarez and Schultz-Cherry 2000a) or using application methods such as GG (Kodihalli *et al.* 1997), are not likely to be applied in the field for routine administration to poultry as they are not practical. Therefore, this new technology for use in poultry is still being developed and improvements in DNA vaccine technology are needed before the vaccines can be made commercially available (Suarez and Schultz-Cherry 2000a).

H6 subtype AI viruses have not only caused sporadic outbreaks of AI in the poultry industry worldwide, but also have been shown to constitute a potential threat to public

health (Chen *et al.* 2009; Myers *et al.* 2007). This highlights the need to develop an H6 vaccine. Live attenuated influenza A virus H6 vaccines generated using the eight-plasmid reverse genetics system were recently developed and evaluated in mice and ferrets (Chen *et al.* 2009). Numerous studies on DNA vaccines have shown that HA-expressing plasmids from H1, H3, H5, H7 and H9 subtypes of influenza A viruses protected mice and chickens against homologous virus challenge (Cherbonnel *et al.* 2003; Kodihalli *et al.* 1999; Kodihalli *et al.* 1997; Kodihalli *et al.* 2000; Lee *et al.* 2003; Ljungberg *et al.* 2002; Ljungberg *et al.* 2000; Qiu *et al.* 2006). To our knowledge, there is no publication detailing similar studies on the efficacy of an H6-DNA vaccine.

This chapter describes investigations of the DNA expression vector VR1012 encoding the HA (VR-HA) or NP (VR-NP) genes of A/coot/WA/2727/79(H6N2) virus with respect to induction of immune responses in chickens, protection from infection and reduction in virus shedding.

3.2 Materials and Methods

3.2.1 Preparation of VR1012 plasmid DNA

The expression vector VR1012 (Vical Inc., CA, USA) (Figure 3.1) and the competent *E. coli* cells DH 5α (Invitrogen) were provided from stocks available in the School of Veterinary and Biomedical Sciences, Murdoch University, Australia.

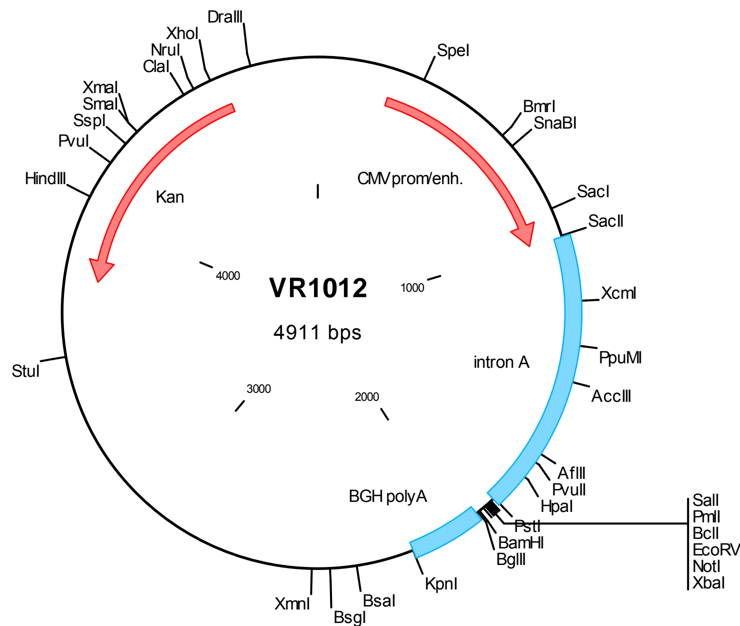

Figure 3.1 Schematic diagram of DNA expression vector VR1012.
(http://www.vical.com/).

3.2.1.1 Preparation of electrocompetent cells

The competent cells were prepared according to the protocol by Dower *et al.*(1988). Briefly, a fresh colony of DH 5α bacterial cells was inoculated into 10 mL SOB medium (2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl) and incubated with vigorous aeration at 250 rpm overnight at 37°C. The overnight culture was diluted into 1 L pre-warmed SOB medium and incubated at 37°C with vigorous aeration at 250 rpm to an optical density at 600 nm wavelength (OD_{600}) of 0.8. The culture was transferred into a chilled sterile centrifuge tube and centrifuged in a cold rotor at 3,000 g for 10 min. The pellets were gently resuspended in 1 L of 4°C cold sterile 10% glycerol. The tube was centrifuged as above. The pellets were resuspended in 0.5 L of

cold sterile 10% glycerol. Following further centrifugation, the pellet was resuspended in 2-3 mL of 10% glycerol. The cells remained on ice and the suspension was dispensed in aliquots of 50 μL/ tube and frozen on dry ice. The cell concentration (approximately 1 x 10^{10} cells/mL) was measured by a spectrophotometer. Finally the cells were stored at -70 °C until required.

3.2.1.2 Transformation of plasmid DNA into *E. coli* by electroporation

The Gene Pulser (Bio-Rad, NSW, Australia) was used to perform transformation as per the instructions (Dower *et al.* 1988). The Gene Pulser (Bio-Rad) was set at 25 μF capacitor, 2.5 kV, and 200 Ω. One microlitre of VR1012 stock plasmid DNA was added into a thawed 50 μL tube of DH 5α cells followed by transfer into a chilled, 0.2 cm electroporation cuvette and insertion into the safety chamber. A pulse of 12.5 kV/cm with a time constant of about 4.0 msec was used. Following removal of the cuvette from the chamber, 1 mL of pre-cooled SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM MgSO4, 20 mM glucose) was quickly but gently added to resuspend the cells. The cell suspension was subsequently transferred to a sterile polypropylene tube and incubated, with shaking at 225 rpm, for about 1 hr at 37°C. Then 10-100 μL of transformed DH 5α culture was spread on 1.2% agar LB plates supplemented with 50 μg/mL kanamycin. The plate was incubated at 37°C overnight until colonies were formed.

3.2.1.3 Identification of VR1012

Single colonies were picked from the plate, inoculated into 5 mL LB for incubation at 37°C overnight. The plasmid DNA was extracted using QIAprep®Spin Miniprep kit

(Qiagen) as described in section 2.2.15.

The presence of plasmid DNA was confirmed by *Hin*dIII digestion as follows: 5 μL of 10 x Tango buffer (Fermantas), 1 μg plasmid DNA, 0.5 μL *Hin*dIII (10 U/μL) and dH_2O were added to give a final volume of 50 μL. Following incubation at 37°C for 2-3 hr, the digestion products were visualised by agarose gel electrophoresis as described in section 2.2.11.

3.2.2 Subcloning of viral HA and NP genes into expression vectors

The recombinant pGEM-T vectors (pT-HA, pT-NP) containing the full-length HA or NP gene of A/coot/WA/2727/79 (H6N2) were described in Chapter 2. The full coding sequences of HA or NP genes were subcloned from the pT-HA or pT-NP into the expression vector VR1012 with *Pst*I or *Xba*I and *Bam*HI restriction enzymes used to generate the final plasmids VR-HA or VR-NP that were used for immunizations.

3.2.2.1 Restriction endonuclease reaction

A 50μL restriction endonuclease reaction mixture consisting of 5 μL of 10 x Tango buffer (Fermantas), 1 μg pT-HA DNA or VR1012 DNA, 1 μL *Bam*HI (10 U/μL), 1 μL *Pst*I (10 U/μL) and dH_2O added up to 50 μL was prepared and incubated at 37°C for 2-3 hr. Similarly, another reaction mixture of 5 μL of 10 x Tango buffer (Fermantas), 1 μg pT-NP or VR1012 plasmid DNA, 1 μL *Bam*HI (10 U/μL), 1 μL *Xba*I (10 U/μL) and dH_2O added up to 50 μL was prepared and incubated at 37°C for 2-3 hr. After checking for complete digestion by agarose gel electrophoresis, the digestion products containing HA or NP genes were fractionated and recovered as per section 2.2.11 and 2.2.13.

3.2.2.2 Ligation, transformation and screening

The ligation was performed as per section 2.2.14.1 using pGEM-T Easy vector kit. The molar ratio of the gene of interest versus vector was approximately 3:1 to 5:1. Transformation was performed according to section 3.2.1.2 (above) and screening of positive clones was conducted as per section 2.2.14.3. The VR-HA or VR-NP constructs were purified using Qiagen columns and sequenced to verify the fidelity of the sequences as per the procedures described in section 2.2.15.

3.2.3 Large-scale preparation of plasmid DNA for use as a DNA vaccine

A single colony of the VR-HA or VR-NP construct was inoculated into 10 mL LB with 50 μg/mL kanamycin and kept at 37°C overnight with shaking at 250 rpm. The following day, 1 ml fresh culture was inoculated into 1L LB with 50 μg/mL kanamycin and incubated at 37°C overnight with shaking at 220 rpm. The bacterial cells were harvested by centrifugation at 2,500 g for 10 min at 4°C. The pellet was fully resuspended in 100 mL NTE buffer (100 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA(pH 8.0)) followed by centrifugation at 3,500 g for 10 min at 4°C. The pellet was resuspended in 50 mL pre-cooled solution I (25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 0.9% (w/v) glucose). This was followed by addition of 100 mL of freshly prepared solution II (0.2 M NaCl, 1%SDS), gently mixing and holding on ice for 10 min. Then 75 mL high salt neutralization buffer (2.5M KOAc, 5% (v/v) formic acid) was added to the mixture and thoroughly mixed until a white precipitate was observed. After centrifugation at 3,500 g for 15 min at 4°C, the supernatant was filtered through four layers of sterile gauze before the addition of an equal volume of isopropanol. The suspension was centrifuged at 2,500 g for 15 min at 4°C. The pellet was resuspended in 15 mL dH_2O and an equal volume of

LiCl solution (5M LiCl, 1% (w/v) 3-(N-Morpholino) propanesulfonic acid (MOPS), pH to 8.0, kept at 4°C) was added. The mixture was kept on ice for 10 min before centrifugation at 3,500 g for 15 min at 4°C. The supernatant was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by centrifugation at 1,900 g for 5 min at 20°C. The aqueous phase was transferred to a polypropylene tube followed by the addition of an equal volume of isopropanol. The mixture was centrifuged at 2,500 g for 15 min at 4°C and the pellet was dissolved in dH_2O followed by the addition of 10 μL RNase A (10 mg/mL, Fermantas) at 37°C for 30 min. The DNA was precipitated by addition of 1/10 volume 3 M NaOAc (pH 4.8) and 2.5 volumes 100% ethanol followed by incubation at -20°C for overnight and centrifugation at 15,000 g for 20 min at room temperature. The pellet was washed in 75% ethanol by centrifugation at 15,000 g for 5 min at room temperature. The DNA pellet was dried briefly, resuspended in dH_2O and stored at -20°C until required. DNA concentration was determined by spectrophotometric analysis at 260 nm and 280 nm.

3.2.4 *In vitro* expression of DNA plasmid in eukaryotic cells

Cos-7 (African green monkey kidney) cells, supplied by the Department of Microbiology, University of Western Australia, were grown as per section 2.2.4.1. Approximately 60-80% confluenct Cos-7 cells were used for transfection. An aliquot of 30 μg plasmid DNA was added to 0.5 mL of 0.25 M $CaCl_2$ followed by dropwise addition to 0.5 mL 2xBBS (50 mM BES(N,N-bis(hydroxyethyl)-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na_2HPO4, pH 6.95) with aeration using a glass pasteur pipette attached to a pipette-man. After incubating for 10 min at room temperature, the DNA mixture was trickled over the cells and the flask was incubated overnight at 37°C. The control flask was

similarly inoculated with the 0.25 M $CaCl_2$/2xBSS mixture without plasmid DNA. After 18 hr the media was removed and the cells were washed twice using about 5 mL of DMEM with 10% FCS. Following the addition of 5 mL DMEM with 10% FCS, the flasks were incubated at 37°C for another 48 hr. After discarding the supernatant the cells were harvested by freezing (-20°C) and thawing, and resuspended in 200-500 μL PBS for Western blotting. Similarly, Cos-7 cells transfected with 200 μL plasmid DNA mixture in 6-well plates were used to conduct IFT using fluorescent microscope (Olympus BH-2, Tokyo, Japan) as per section 2.2.5.

3.2.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB)

3.2.5.1 SDS-PAGE

Protein samples were analysed by SDS-PAGE on discontinuous gels (Laemmli 1970). One dimensional gel electrophoresis was conducted under reducing conditions using a mini-protein electrophoresis apparatus (Bio-Rad). A 12.5% resolving gel (5 mL) containing 2.17 mL dH_2O, 1.25 mL of 1.5 M Tris (pH 8.8), 0.05 mL of 10% SDS, 1.5 mL of 40% acrylamide/bis (37.5:1), 25 μL of 10% ammonium persulfate (APS) and 5 μL TEMED was prepared in the gel apparatus, followed by addition of 0.5 mL dH_2O or butanol to level the resolving gel surface. Once the resolving gel became solid, a 4% stacking gel (2.5 mL) containing 1.58 mL dH_2O, 630 μL of 0.5 M Tris (pH 6.8), 25 μL of 10% SDS, 250 μL 40% acrylamide/bis, 12.5 μL of 10% APS and 2.5 μL TEMED was then added, followed by insertion of an electrophoresis comb to create loading wells. Prior to electrophoresis, transfection product was mixed with an equal volume of 2x loading buffer (2 mL dH_2O, 0.5 ml 0.5 M Tris pH6.8, 400 μL glycerol, 800 μL of 10%

SDS, 200 μL β-mercaptoethanol and 100 μL 0.05% bromophenol blue) followed by boiling for 10 min. Then 20 μL samples were added to the wells and the electrophoresis was conducted at 150 V for 1.5-2 hr at room temperature in running buffer (2.9 g/L Tris base, 14.4 g/L glycine, and 1 g/L SDS).

3.2.5.2 Coomassie blue staining

Following electrophoresis, the protein bands were visualized by staining with Coomassie brilliant blue dye at room temperature overnight, with rocking. The gel was destained for about 2 hr in a solution containing 10% acetic acid and 40% methanol, and destaining was stopped with 7% glacial acetic acid when protein bands were clearly evident.

3.2.5.3 Western blotting

The separated protein bands were transferred from gels onto Hybond™ nitrocellulose membrances (Bio-Rad) using a mini trans-blot apparatus (Bio-Rad). Transfer was performed in transfer buffer (3.03 g Tris base, 14.4 g glycine, 200 mL methanol per L) overnight at 30 V at 4°C. The membrane was blocked in PBST containing 5% (w/v) skim milk powder for 1 hr at room temperature. Chicken anti-H6 hyperimmune serum at a dilution of 1:100 was added to the membrane followed by incubation at room temperature for 1 hr. After washing 3 times with PBST (5 min per time), the membrane was incubated with horseradish peroxidase (HRP) conjugated anti-chicken immunoglobulin (Ig) (Sigma) at a dilution of 1:500 for 1 hr at room temperature. Following washing 3 times in PBST, the colour was developed with a 4-chloronaphthol (4-CN) substrate solution (100 mL of TBS [0.02 M Tris base, 0.385 M NaCl (pH 7.5)], 60 μL H_2O_2, 20 mL methanol, and 60 mg 4-CN (Bio-Rad) at room temperature for 10

min as described by the manufacturer. After the colour developed, the reaction was stopped by rinsing the membrane in dH_2O and the membrane was then allowed to dry.

3.2.6 Measurement of antibodies to H6 avian influenza A proteins

3.2.6.1 HI test

The HI tests were conducted on serum from vaccinated and control chickens using the procedures described in section 2.2.3.3.

3.2.6.2 Enzyme-linked immunosorbent assay (ELISA)

3.2.6.2.1 Indirect ELISA

ELISA antigen was prepared as described previously (Fatunmbi *et al.* 1989; Johansson *et al.* 1989; Qiu *et al.* 1992). Briefly, infective allantoic fluid was centrifuged at 3,000 g for 20 min and the supernatant was then centrifuged at 180,000 g for 2 hr at 4°C. The pellet was resuspended in NTE buffer. The concentrated virus was centrifuged through 30% sucrose onto a 60% sucrose cushion and at 160,000 g for 1.5 hr at 4°C. Virus was removed from the interface and diluted 1:5 in NTE buffer. Following centrifugation at 150,000 g for 1 hr at 4°C, the pellet was suspended in a small volume of NTE buffer. The concentrated virus was inactivated by adding a final concentration of 0.5% SDS for 60-min incubation at 37°C. This ELISA antigen was assayed for protein concentration by a spectrophotometer and stored at -70°C. Similarly, a negative antigen was prepared from SPF chicken embryonated allantoic fluids.

Checkerboard titrations were performed using different antigen, control serum and conjugate concentrations to determine the optimal antigen concentration, and conjugate and serum dilutions. The indirect ELISA procedure was as follows: The 96-well ELISA

microplates (Greiner Bio-one) were coated with 100 µL per well of antigen diluted in 0.05 M carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4°C overnight. After washing three times (5 min per time) with PBST, 100 µL blocking solution (PBST containing 5% skim milk powder) was added followed by incubation at 37°C for 30 min. Then 100 µL of diluted test sera, anti-H6 positive control serum or SPF chicken serum were added followed by incubation at 37°C for 30 min. Following washing, 100 µL of diluted HRP-conjugated rabbit anti-chicken IgG (Chemicon International Inc., Melbourne, Australia) was added and the plate was incubated at 37°C for 30 min. After the last washing 100 µL of TMB substrate (Promega) was added and plates were incubated at room temperature for 5 min, when the reaction was stopped by adding 50 µL of 2 M H_2SO_4. The optical density (OD) was read at 450 nm using a Multimode detector (DTX880 Beckman Coulter Inc., CA, USA). Results were expressed as a test to negative (T/N) ratio equal to the OD of the test sample divided by the OD of negative control; with T/N ratio over 2.0 considered as positive.

3.2.6.2.2 Competitive ELISA (c-ELISA)

Competitive ELISA reagents and test procedures were supplied by Dr Paul Selleck, CSIRO, AAHL, Geelong, Australia. Competitive Influenza type A ELISA was performed as follows: ELISA plates (Greiner Bio-one) were coated with 50 µL of influenza A nucleoprotein antigen per well in carbonate-bicarbonate coating buffer diluted as directed followed by incubation for 1 hr at 37°C on a microplate shaker at a speed of 400 rpm. After washing 3 times with PBST, 50 µL of test serum (diluted 1:10 in sample diluent (PBST containing 1% skim milk powder) was added to appropriate wells. The negative serum diluted 1:10 and positive serum diluted 1:10 and 1:100 as the high and

low positive controls, as well as monoclonal antibody (mAb) control (sample diluent only) were added respectively. Subsequently, 50 µL mAb that specifically binds AIV nucleoprotein diluted as directed, was added followed by incubating at 37°C for 30 min on a plate shaker. After washing, 50 µL of optimally diluted anti-mouse IgG HRP conjugated antibody (Silenus, VIC, Australia) was added followed by incubating at 37 °C for 30 min on a plate shaker. Following washing, 50 µL TMB substrate were added and incubated for 5 min with shaking followed by 50 µL of 1 M H_2SO_4 stopping solution. The OD results were read on a multiscan ELISA reader at 450 nm and then calculated as the percentage inhibition of binding of mAb in the absence of any serum. The test was acceptable if OD of mAb average was more than 0.8, and inhibition percentage of high positive, low positive and negative control were more than 80%, more than 40%, and less than 40%, respectively. The results were calculated using the following formula (Av refers to average):

$$\% \text{ inhibition} = 100 - \left(\frac{(\text{Av OD of sample}}{\text{Av OD of mAb}} \times 100 \right)$$

Test sera giving inhibitions of less than 40% were negative, and those giving greater than 60% inhibitions were positive. Sera giving inhibitions of between 40% to 60% were considered equivocal.

The indirect ELISA was used to detect antibody in VR-HA vaccinated chickens and the competitive ELISA was used for the detection of antibody in VR-NP vaccinated chickens.

3.2.6.3 Immunoblot assay

The immunoblot assay was performed as described for WB in section 3.2.5.3 above. The above-mentioned inactivated purified virus was equally mixed with non-reducing loading

buffer (2% SDS, 20% sucrose, 0.01% (v/v) bromophenol blue, 0.125M Tris (pH 6.8)) followed by boiling for 5 min (Qiu *et al.* 1992). The virus samples were applied to an SDS-PAGE gel in a single gel wide well, producing a continuous band of antigen across the gel. Protein bands in the unstained SDS-PAGE gel were then transferred to Hybond^{TM} nitrocellulose membrances. The blotted membrane was then cut in longitudinal strips and WB was performed on strips using sample sera (including positive and negative control antisera) at appropriate dilutions, followed by anti-chicken IgG, HRP conjugate and developed with Bio-Rad colour development reagent.

3.2.7 Preparation of inactivated H6N2 vaccine as a control vaccine

HA antigen was prepared as per section 2.2.2. To mimic currently used commercial water-in-oil (W/O) AI vaccine formulations for poultry, the oil-based adjuvant Montanide^{TM} ISA-70 VG (Seppic, Paris, France) was used. According to the manufacturer's recommended protocols, 2.6 mL inactivated undiluted allantoic fluid (HA titre 2^7) was added dropwise into 7.4 mL Montanide^{TM} ISA-70 VG adjuvant by stirring with magnetic stirrers and mixed for 5-6 hr until a W/O emulsion was formed.

3.2.8 Preparation of DNA vaccine formulations

3.2.8.1 DNA vaccine

Various dilutions containing the required quantity of plasmid DNAs (as shown in Tables 3.1 and Table 3.2) were made in sterile PBS (pH 7.2) or 0.9% NaCl solution as described below.

3.2.8.2 DNA vaccine with lipofectin adjuvant

For the DNA vaccines using lipofectin as adjuvant 20 μL lipofectin (Invitrogen) was mixed with 100 μL PBS and kept for 30 min at room temperature to form liposomes as described (Lee *et al.* 2006). As indicated in Table 3.1 and 3.2, various concentrations of plasmid DNAs were diluted into a total volume of 280 μL and then mixed with the liposome. The plasmid-liposome mixture was allowed to stand for 15 min at ambient temperature before injection.

3.2.9 DNA immunization of chickens

The chickens were 6-week-old layer pullets (Hy-Line) supplied by Altona Hatchery Pty. Ltd., Australia.

3.2.9.1 Experiment 1

Birds were housed in steel mesh cages with one bird per cage and fed commercial poultry feed and water *ad lib* at the animal house, Murdoch University, Australia. The immunization protocol is shown in Table 3.1. Four groups of five 6-week-old chickens were all intramuscularly injected twice, 3 weeks apart, with the appropriate amount of VR-HA or blank vector (VR) plasmid DNA dissolved in 0.2 mL 0.9% NaCl, with a 0.1 mL injection in each leg. The control group was intramuscularly injected with 1 mL of inactivated H6N2 virus vaccine (IVV). All the chickens were shifted to an AEC-approved free-range pen at Jandakot, Western Australia, 2 weeks after the second injection.

Six weeks after the second injection, three chickens from each group were randomly selected to receive a third IM injection of their respective dose of plasmid DNA plus

lipofectin adjuvant. Seventeen weeks later, selected groups received a fourth vaccination using the same regimen as the third one.

Chickens were bled by venepuncture from the wing veins at the initial vaccination and at intervals of 2 weeks post-vaccination to measure the H6-specific antibodies using HI, ELISA and immunoblotting assay.

Homologous virus challenge was conducted 30 weeks following the last vaccination for chickens that did not receive booster vaccination with lipofectin adjuvant (non-adjuvanted chickens) and 7 weeks following the last vaccination for those receiving booster vaccinations with adjuvant (adjuvanted chickens).

3.2.9.2 Experiment 2

A second vaccination experiment was conducted using a similar immunization regimen as experiment 1. Each group of 3 SAN chickens, reared at an AEC-approved free-range pen at Jandakot, Australia, was intramuscularly administered VR-HA, VR-NP or VR plasmid DNA in 0.2 mL PBS, with or without lipofectin adjuvant, at the doses indicated in Table 3.1. Three weeks later the birds received a second injection of the same dose and then a third injection was given 6 weeks later. The birds were bled and tested as described for experiment 1. Selected groups were subjected to virus challenge at 7 weeks following the last vaccination.

3.2.10 Virus challenge and virus isolation

Virus challenge was performed as per section 2.2.8 using $EID_{50}10^{7.25}$/0.1 mL H6N2 virus. OS and CS were collected daily from the chickens. Serum was separated from the collected blood for further serological testing.

Table 3.1 Number of birds used in experiment 1 and experiment 2.

	Experiment 1								Experiment 2							
Vaccine type	VR	IVV	VR-HA						VR	VR-HA			VR-NP			
Dose (μg)	300		50		100		300		100[a]	100[a]	500	500[a]	100	100[a]	500	500[a]
1st, 2nd vaccination	5	5	5		5		5		3	3	3	3	3	3	3	3
3rd vaccination	3[a]		3[a]			3[a]	3[a]		3	3	3	3	3	3	3	3
4th vaccination	3[a]		3[a]				3[a]									
Virus challenge	4[b]	5	3	2	1		3	2	3	3	1	3		3		3
Group for analysis	1	6	2	4	4		3	5	11	7	5	8		9		10

a - Adjuvant; b - One bird died.VR, IVV, VR-HA and VR-NP refer to the empty vector VR1012, inactivated H6N2 virus vaccine, HA-expressing construct, NP-expressing construct respectively.

Virus isolation was performed as per section 2.2.9. The virus titre in selected positive samples was measured by serial dilution and inoculation in chicken embryos as per 2.2.6.

3.2.11 Data analysis

Statistical analyses were conducted as per section 2.2.17.

3.3 Results

3.3.1 *In vitro* expression of the VR-HA and VR-NP constructs

Restriction enzyme analysis of the inserted of HA or NP genes in VR1012 vector showed inserts of expected size (1.7 kb for HA and 1.6 kb for NP) (Figure 3.2). The expression of recombinant VR-HA or VR-NP in Cos-7 cells was confirmed by WB (Figure 3.3). As shown in Figure 3.3, the intensity of approximately 66 kD (HA protein) or 56 kD (NP protein) bands became correspondingly darker when the dose of transfected plasmid DNA was increased from 20 μg to 40 μg. No specific band was observed at the corresponding site in both Cos-7 and blank vector controls. The expression of VR-HA or VR-NP in Cos-7 cells was also confirmed by IFT (Figure 3.4). Compared to the blank vector control, bright fluorescence in the cytoplasm was observed in both HA and NP vector transfected cells. No specific fluorescence was observed in the blank vector control.

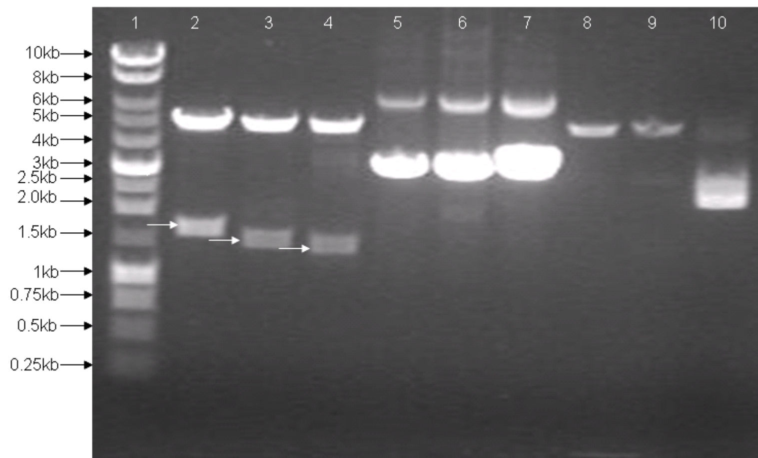

Figure 3.2 Identification of insert genes in VR1012 vector.

Lane 1, 1 kb marker; lane 2, HA (*Bam*HI/*Pst*I); lane 3, NP (*Bam*HI/*Xba*I); lane 4, NA (*Bam*HI/*Xba*I); lane 5, HA (undigested); lane 6, NP (undigested); lane 7, NA (undigested); lane 8, VR1012 (*Bam*HI/*Pst*I); lane 9, VR1012 (*Bam*HI/*Xba*I); lane 10, VR1012 (uncut) (Arrow represents HA, NP and NA, respectively).

Figure 3.3 Transient expression of recombinant HA and NP in Cos-7.

Lanes 1-3, 20, 30, 40 µg VR-HA plasmid DNA; lane 4, virus control; lane 5, Cos-7; lane 6, VR1012; lane 7, Marker (Bio-Rad, 250, 150, 100, 75, 50, 37 kD, from top to bottom); lanes 8-10, 40, 30, 20 µg VR-NP DNA. Arrow refers to specific band.

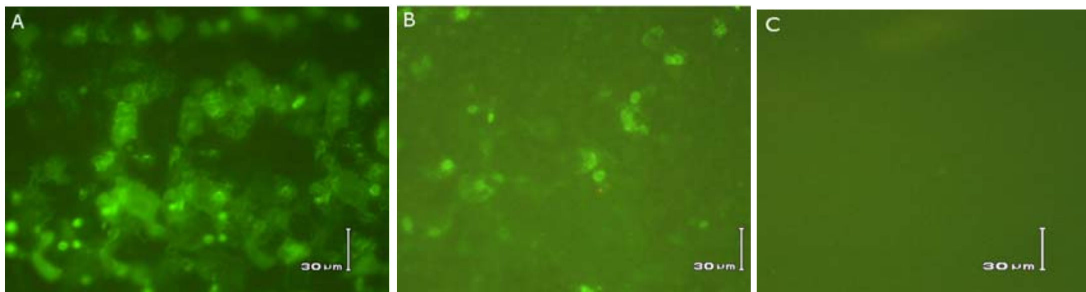

Figure 3.4 Expression of HA and NP in transfected Cos-7 cells by IFT.

A, VR-HA; B, VR-NP; C, blank vector control (x10).

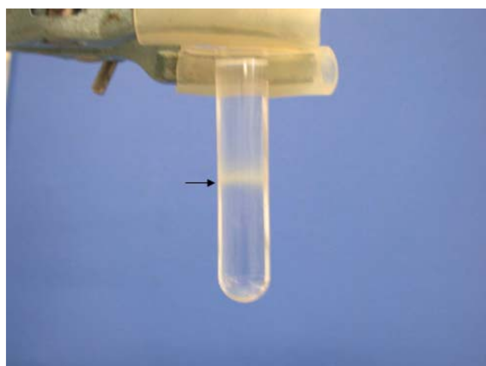

Figure 3.5 Virus band indicated by arrow following sucrose gradient ultracentrifugation.

3.3.2 Optimal parameters of indirect ELISA

ELISA antigen was prepared from the collected virus band (Figure 3.5). The graphs showing optimization of ELISA parameters are shown in Figure 3.6. Positive/negative (P/N) ratio was the optical density at 450 nm wavelength (OD_{450}) from virus antigen coated wells divided by the OD_{450} of the negative antigen wells. ELISA plates were coated with different concentrations of virus or negative control antigen and a checkerboard titration was performed with different dilutions of conjugate. The 50 ng/well antigen showed higher P/N ratio at the conjugate dilution of either 1/2000 or 1/4000. Dilutions of H6-specific chicken antiserum were reacted against different antigen concentrations. The higher P/N ratio was under 50 ng/well antigen and serum dilution of 1/100 or 1/200. Thus, the optimal ELISA parameters that were used for subsequent testing were 50 ng/well (0.5 ng/μL) antigen, serum at a dilution of 1/200, conjugate at a dilution of 1/4000.

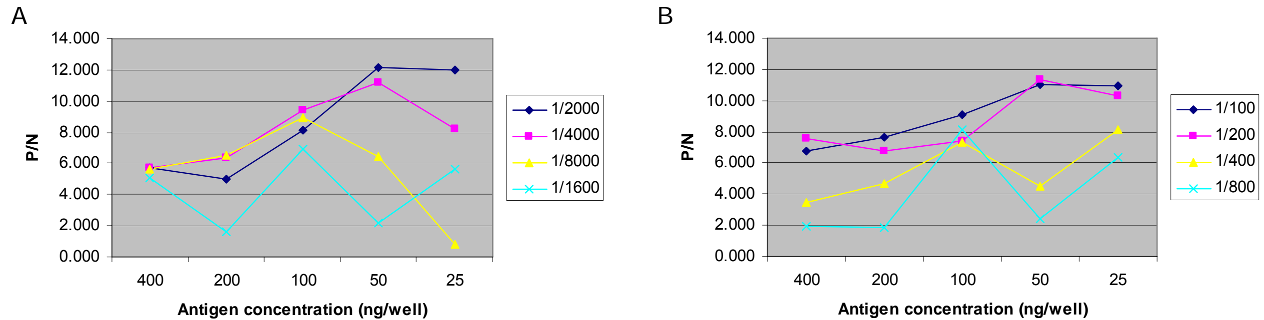

Figure 3.6 Optimization of ELISA parameters. P/N ratio refers to the optical density from virus antigen coated wells divided by the OD_{450} of the negative antigen wells.
A Different conjugate dilution versus different antigen concentration under constant serum dilution. **B** Different antigen concentration versus different serum dilution under fixed conjugate dilution.

3.3.3 Effect of different doses and vaccination times on VR-HA antibody response

3.3.3.1 Antibody response elicited by VR-HA in experiment 1

The chickens vaccinated twice 3 weeks apart with either the 50, 100 or 300 µg dose of VR-HA elicited no measurable antibody using HI, ELISA and immunoblotting assays during the 6 week period post-vaccination. One remaining bird from the100 µg group transiently showed a low HI titre (2^1) at 24 weeks following the second vaccination.

In an attempt to induce an antibody response by VR-HA, three chickens from the 50 µg, 100 µg and 300 µg dose groups above were randomly selected to be given a booster with VR-HA plus lipofectin as adjuvant, using the same dose and delivery route. As shown in Table 3.2, only one chicken (identification number 50A in Figure 3.7) from the 50 µg group produced a low level of HI antibody (titre 2^2) and this was confirmed by immunoblotting assay and ELISA. The highest immunoblotting titres for chicken 50A were at 2 weeks post-vaccination with a titre of 1/320 and at 4 weeks with a titre of 1/640 after three vaccinations (Figure 3.7). Another chicken (100A) from the 100 µg group

gave a low HI antibody titre (2^2) but this could not be confirmed by immunoblotting assay. No HI and immunoblotting antibody in any other vaccinated chickens was observed over the 17-week period following three vaccinations.

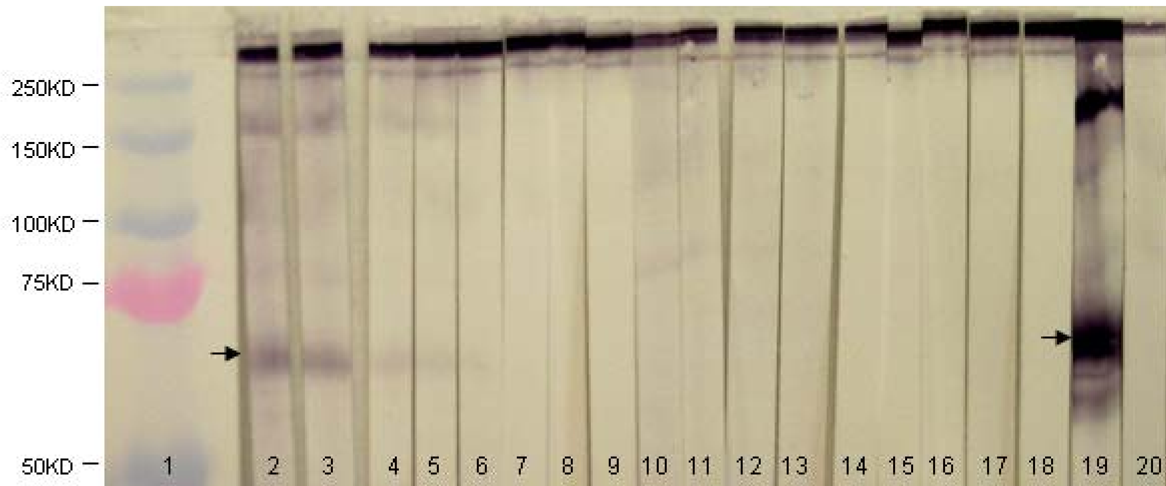

Figure 3.7 Antibody detection by immunoblotting assay.
Lane1, marker (Bio-Rad), Lanes 2-9, VR-HA vaccinated chicken 50A serum 4 weeks post-vaccination at dilutions 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280,1/2560, 1/5120 dilution; Lanes 10-18, VR1012 vector control vaccinated chicken serum 4 weeks post-vaccination at dilutions of 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280 dilution; Lane 19, H6 positive serum at 1/125 dilution; Lane 20, pre-vaccination serum at 1/5 dilution.

In an attempt to further enhance the antibody response, 17 weeks following the three vaccinations, the 50 µg and 300 µg dose groups were boosted a fourth time with their respective dose of VR-HA with lipofectin adjuvant, identical to the third vaccination. As shown in Table 3.2, a total of three out of six chickens that received the VR-HA vaccine generated detectable HI antibody. One chicken from the 50 µg group that showed a low HI titre following three vaccinations had a HI titre of 2^8 after four vaccinations, which was confirmed by immunoblotting assay. The other two from each of the 50 µg and 300 µg group showed lower HI titres (2^2).

Table 3.2 Antibody conversion in chickens immunized with VR-HA construct in experiment 1 and 2.

Experiment 1						Experiment 2							
Vaccine type	VR	IVV	VR-HA			VR	VR-HA			VR-NP			
Dose (µg)	300		50	100	300	100[a]	100[a]	500	500[a]	100	100[a]	500	500[a]
2nd vaccination	0/5[b]	5/5	0/5	1/5[c]	0/5	0/3	0/3	0/3	0/3	1/3[d]	0/3	0/3	0/3
3rd vaccination	0/3		1/3[a]	0/3	0/3 [a]	0/3	1/3	0/3	1/3[e]	0/3	0/3	0/3	0/3
4th vaccination	0/3		2/3 [a]	ND[f]	1/3 [a]								

a - Adjuvant
b - Number of sero-conversions / total number of vaccinated chickens
c - Antibody appeared at 24 weeks following the second vaccination
d - Competitive ELISA
e - Immunoblotting antibody
f - Not done.

3.3.3.2 Antibody responses post virus challenge in experiment 1

All vaccinated chickens sero-converted by 10 days after virus challenge and the antibody range within the groups were shown in Table 3.3. There was no significant difference in GMT of HI antibody between pre- and post-virus challenge in the blank vector group (Group 1), Group 2 (50 µg VR-HA group with adjuvant) or Group 5 [300 µg (2 birds) or 500 µg (1 bird) VR-HA without adjuvant] but statistically significant differences in Group 3 (300 µg VR-HA group with adjuvant)(P=0.044), Group 4 [50 µg (2 birds) and 100 µg (1 birds) VR-HA without adjuvant] (P=0.028) and Group 6 (inactivated vaccine group) (P=0.033). Moreover, there was no significant difference in HI GMT before or after virus challenge between any of the VR-HA groups and the blank vector control group.

Table 3.3 HI antibody titre and statistical analysis pre-challenge (PRC) and post-challenge (POC) for vaccine groups in experiment 1

	Group 1*	Group 2	Group 3	Group 4	Group 5	Group 6
PRC	0**^{Aa}	$2^{0\text{-}8}(2^{3.3})^{Aab}$	$2^{0\text{-}2}(2^{0.7})^{Aa}$	$2^{0\text{-}2}(2^{0.7})^{Aa}$	0^{Aa}	$2^{4\text{-}7}(2^{6})^{Ab}$
POC	$2^{3\text{-}8}(2^{5.7})^{A\ a}$	$2^{7\text{-}7}(2^{7})^{Aa}$	$2^{5\text{-}10}(2^{7.7})^{Bab}$	$2^{8\text{-}13}(2^{10.3})^{Bab}$	$2^{3\text{-}10}(2^{6.3})^{Aab}$	$2^{8\text{-}12}(2^{9.6})^{Bb}$

*Groups 1, 2 and 3 were vaccinated with either 300 µg blank vector plasmid DNA, 50 µg VR-HA or 300 µg VR-HA respectively with two vaccinations without adjuvant followed by two vaccinations with adjuvant. Group 4, 50 µg VR-HA (2 birds), and 100 µg VR-HA (1 birds) given two vaccinations without adjuvant. Group 5, 300 µg VR-HA (2 birds) given two vaccinations and one bird given 500 µg VR-HA for three vaccinations. Group 6, inactivated virus vaccine given two vaccinations.
** HI titre for the groups shown as range with GMT.
Within the column for Groups 3, 4 and 6 the different uppercase superscript letter indicates statistical differences ($P < 0.05$) using the Paired-sample T test. There were no significant differences in GMT between pre- and post-challenge for the other groups.
For the row groups with different lowercase superscript letter are significantly different ($P < 0.05$) using ANOVA or Mann-Whitney test. Except for significant difference between Group 6 and Group 1 or 2, there were no significant differences in HI GMTin other groups after virus challenge.

The GMT of Group 6 pre-challenge was significantly higher than the blank vector controls (Group1) and three of the four VR-HA groups (Groups 3, 4 and 5), but Group 6 was no significantly different from Group 2 (50 μg VR-HA with adjuvant) (P=0.45) prior to challenge. However there was a significant difference (P=0.02) in GMT between Group 2 and Group 6 post challenge. In addition, there was a significant difference in GMT pre- and post-challenge between Group1and Group 6.

3.3.3.3 Antibody response induced by VR-HA in experiment 2

In experiment 2, chickens administered 100 μg or 500 μg VR-HA twice with or without lipofectin adjuvant for the initial vaccinations also did not induce measurable antibody. As shown in Table 3.2, following three vaccinations, only one chicken from the group inoculated with the 100 μg VR-HA and the adjuvant produced a low level of HI antibody (2^1), which was confirmed by immunoblotting assay, at 4 weeks after the third vaccination. Another chicken from the 500 μg adjuvanted group did not develop HI antibody but its immunoblotting antibody titre was 1/20. No HI or immunoblotting antibody was detected in any other chickens during the 7 week period following three vaccinations.

3.3.3.4 Antibody responses post viral challenge in experiment 2

In order to assess whether VR-HA developed a protective immune response, six chickens vaccinated with VR-HA and adjuvant were challenged with the homologous virus. As illustrated in Table 3.4, the HI titre in VR-HA vaccinated chickens ranged from 2^5 to 2^{14} in the VR-HA vaccinated groups after virus challenge. There was a significant difference (P=0.009) in the GMT in the adjuvanted 100 μg VR-HA group (Group 7) before and after

challenge but there was no significant difference (P=0.086) in the adjuvanted 500 µg VR-HA group (Group 8) between pre-and post challenge. In the adjuvanted empty vector group (Group 11), there was no significant difference (P=0.081) in the GMT between pre-and post-challenge. Furthermore, there was no statistical difference in the GMT both prior to and after virus challenge between each of the VR-HA groups and the blank vector group.

Table 3.4 HI antibody titre and statistical analysis pre-challenge (PRC) and post-challenge (POC) for vaccine groups in experiment 2.

	Group 6*	Group 7	Group 8	Group9	Group 10	Group 11
PRC	$2^{4-7}(2^{6})^{**\ Aa}$	$2^{0-2}(2^{0.7})^{Ab}$	0^{Ab}	0^{Ab}	0^{Ab}	0^{Ab}
POC	$2^{8-12}(2^{9.6})^{Ba}$	$2^{6-7}(2^{6.7})^{Bbf}$	$2^{5-14}(2^{8.7})^{Aabc}$	$2^{4-6}(2^{4.7})^{Bbc}$	$2^{3-5}(2^{3.7})^{Bc}$	$2^{3-10}(2^{7.7})^{Aacf}$

*Group 6, inactivated virus vaccine given two vaccinations. Group 7and 8, 100 µg and 500 µg VR-HA given three vaccinations with adjuvant respectively. Group 9 and 10, 100 µg and 500 µg VR-NP given three vaccinations with adjuvant respectively. Group 11, 100 µg blank vector plasmid DNA given three vaccinations with adjuvant. ** HI titre for the groups shown as range with GMT.
Within the column the different uppercase superscript letter indicates statistical differences ($P < 0.05$) using the Paired-sample T test. For the row groups with different lowercase superscript letter are significantly different ($P < 0.05$) using ANOVA or Mann-Whitney test.

3.3.3.5 Antibody response induced by VR-NP

Following VR-NP vaccination, three out of 12 VR-NP vaccinated chickens (one weak positive and 2 equivocal) induced a weak antibody response to the NP of the H6 virus by cELISA. One chicken from the 100 µg VR-NP group without lipofectin gave a weak positive result using cELISA at 4 weeks after the second vaccination (Table 3.2), but a equivocal ELISA result at 4 weeks following the third injection. One bird from the adjuvanted 500 µg VR-NP group showed an equivocal result by cELISA following the second injection. Another chicken from the adjuvanted 100 µg VR-NP group showed a equivocal result by cELISA after three vaccinations. No antibody was detected in any other vaccinated chickens for the 7 week period following three vaccinations.

As shown in Table 3.4 following virus challenge, HI titre in six chickens vaccinated with adjuvanted VR-NP showed a range of HI titres from 2^3 to 2^6 with an average of $2^{4.17}$. There was significant difference (P<0.05) in GMT between pre- and post-challenge in either 100 µg or 500 µg VR-NP groups. However, in comparison with the blank vector group, there was no statistical difference in GMT in both pre- and post-challenge in either 100 µg or 500 µg VR-NP group.

In addition, there was no significant difference (Mann-Whitney Test, P=0.32) before virus challenge but a significant difference (ANOVA, P=0.031) post-challenge between the combined VR-HA groups and the combined VR-NP groups in experiment 2.

3.3.3.6 Effect of lipofectin adjuvant on numbers of chickens showing seroconversion

As illustrated in Table 3.2, three out of nine chickens that received the VR-HA and adjuvant and one out of six chickens that received the VR-HA alone showed sero-conversion in experiment 1. Also two out of 12 chickens vaccinated with the VR-HA or VR-NP plus adjuvant and one out of nine chickens that received the VR-HA or VR-NP alone showed sero-conversion in experiment 2. Overall, there were seven sero-conversions out of 36 vaccinated birds, with five seroconversions out of 21 from groups given DNA vaccines with lipofectin adjuvant and two out of 15 from groups given vaccines without adjuvant, but there was not a significant difference (Fisher's extract test, P=0.67) between the adjuvanted and non-adjuvanted groups.

3.3.4 Effect of DNA vaccines on virus shedding following virus challenge

No clinical signs were observed during 10 days following H6N2 LPAI virus challenge except for one chicken that received two doses of the empty vector that died on day 5

after virus challenge from parasitism associated with a heavy intestinal nematode infection unrelated to the study.

3.3.4.1 Virus shedding in blank vector control group

All swabs from the 7 blank vector vaccinated chickens over the 10 days after challenge were subjected to virus isolation. As shown in Table 3.5, virus shedding occurred intermittently during this period, with a positive rate of 26.15% (17/65) in CS and 47.69% (31/65) in OS respectively. Most virus excretion occurred within the first 7 days following virus challenge. The virus isolation rate in the OS was 65.96% (31/47) from day 1 to day 7 following virus challenge, whereas, the positive rate in CS was 27.66% (13/47) for the same period, indicating that the respiratory tract was an important route for this LPAI virus shedding. As shown in Figure 3.8, the highest titre in OS fell between day 3 and day 4 post-challenge, with the peak titre of 10^3 EID_{50}/0.1 mL.

Table 3.5 Frequency of virus shedding from individual birds in the blank vector control group*.

Day	1a**		1b		1c		1d***		11a		11b		11c	
	OS	CS	OS	CS	OS	CS	OS	CS	OS	CS	OS	CS	OS	CS
Day 1	+	–	+	–	+	–	+	–	+	–	+	–	+	–
Day 2	+	–	+	–	+	–	–	–	+	–	+	–	+	–
Day 3	–	–	+	–	–	–	–	–	+	–	+	+	+	–
Day 4	–	–	+	–	+	+	+	–	+	+	+	+	+	+
Day 5	–	–	+	–	–	–	–	+	+	–	–	+	+	+
Day 6	–	–	+	–	–	+			+	–	–	+	–	+
Day 7	–	–	+	–	–	–			+	–	–	+	+	+
Day 8	–	–	–	–	–	–			–	–	–	+	–	–
Day 9	–	–	–	–	–	–			–	–	–	–	–	+
Day 10	–	–	–	+	–	–			–	–	–	–	–	+

* + refers to positive and – refers to negative for virus isolation. ** Number refers to group number as described in text and in Table 3.1 and individual birds are designated a, b, and d within the groups. *** This chicken died 5 days after virus challenge.

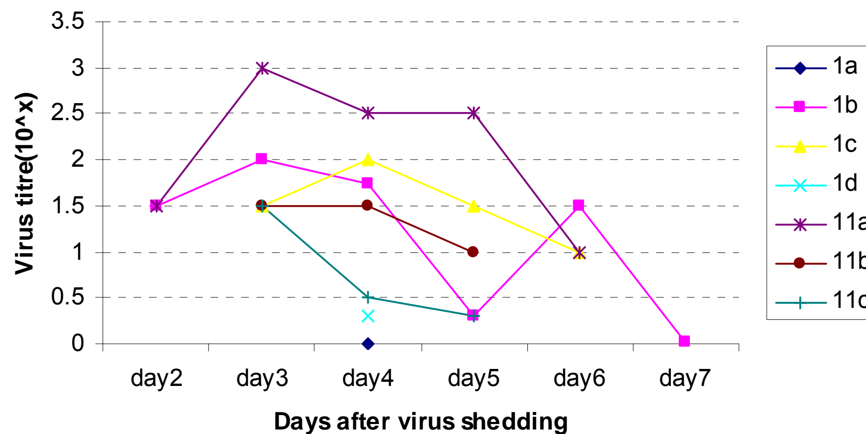

Figure 3.8 Level of virus shedding post-challenge in blank vector groups in experiments 1 and 2.
Number refers to group number as described in text and in Table 3.1 and individual birds are designated a, b, c and d within the groups.

3.3.4.2 Comparison of virus recovery between VR-HA, inactivated vaccine and blank vector groups in experiment 1

The rate of virus recovery in both OS and CS for the VR-HA vaccinated groups, the inactivated H6N2 vaccine group and the blank vector control group is illustrated in Table 3.6. For the OS swabs, there were no significant differences in virus shedding rates between the blank vector controls and any of the four VR-HA groups. However, there was a significant reduction in virus shedding (Fisher's exact tests, $P=0.035$) for the inactivated vaccine group (Group 6) compared with the blank vector group (Group 1) and also compared with the groups vaccinated with VR-HA without adjuvant (Groups 4 and 5) ($P< 0.05$). Group 6 was not significantly different in virus shedding in OS from the 50 µg adjuvanted VR-HA group (Group 2) or 300 µg adjuvanted VR-HA group (Group 3). For the CS results Groups 3 and 4 showed a significantly higher ($P< 0.05$) level of virus shedding compared with the other VR-HA group (Group 2), blank vector controls and

inactivated vaccine groups. There were no significant differences in virus recovery in CS between the other VR-HA groups, the inactivated vaccine group, or the blank vector control group. In terms of virus titre being shed, the virus titre in OS in the VR-HA groups on day 4 after virus challenge was shown in Table 3.7. There was no significant difference in the GMT of virus shedding between Group 2 and Group 3, between Group 4 and Group 5, and between Group1 and any of the four VR-HA groups. However, there was a significant difference between Group 1 and Group 6 (Mann-Whitney Test, P=0.029).

Table 3.6 Virus recovery from VR-HA vaccinated chickens for six consecutive days post-challenge in experiment 1.

	Group 1	Group 2	Group 3	Group 2+ Group3	Group 4	Group 5	Group 4+ Group 5	Group 6
OS	10/22(45.5) [a]	5/18(27.8) [ac]	8/18(44.4) [ac]	13/36(36.1) [ac]	9/18(50.0) [a]	10/18(55.6) [a]	19/36(52.8) [a]	2/18(11.1) [bc]
CS	3/22 (13.6) [acg]	0/18(0) [a]	5/18(27.8) [bcdf]	5/36(13.9) [af]	7/18(38.9) [bcd]	4/18(22.2) [adg]	11/36(30.6) [bfg]	0/18(0) [a]

* Group 1, 2 and 3 referred to 300 µg blank vector plasmid DNA, 50 µg and 300 µg VR-HA respectively with two vaccinations without adjuvant followed by two vaccinations with adjuvant. Group 4, 50 µg (2 birds), and 100 µg (1 birds) VR-HA given two vaccinations without adjuvant. Group 5, 300 µg VR-HA (2 birds) given two vaccinations and one bird given 500 µg VR-HA for three vaccinations. Group 6, inactivated virus vaccine given two vaccinations.

**HA positive numbers/total sample numbers. Percentages are shown in parenthesis.

For the row groups with different lowercase superscript letter are significantly different ($P < 0.05$) using Chi-square or Fisher's exact tests.

Table 3.7 Peak virus titre in OS in individual chickens on day 4 post-challenge.

No. Bird	VR-HA						VR-NP		VR-NP		IVV
	Group2*	Group3	Group4	Group5	Group 7	Group 8	Group 9	Group10	Group1	Group11	Group 6
1	10^{1}	<1**	$10^{1.5}$	$10^{1.5}$	$10^{0.03}$	<1	$10^{0.03}$	10^{1}	$10^{1.75}$	$10^{2.5}$	<1
2	<1	<1	$10^{0.03}$	$10^{0.5}$	<1	$10^{0.5}$	$10^{1.5}$	<1	10^{2}	$10^{0.5}$	<1
3	$10^{0.3}$	$10^{0.5}$	$10^{0.3}$	10^{1}	10^{2}	$10^{1.5}$	$10^{2.5}$	$10^{1.5}$	$10^{0.3}$	$10^{1.5}$	<1
4									<1		<1
5											<1
GMT	$10^{0.44}$	$10^{0.17}$	$10^{0.61}$	10^{1}	$10^{0.68}$	$10^{0.67}$	$10^{1.34}$	$10^{0.83}$	$10^{1.35}$	$10^{1.5}$	$10^{0.001}$

* Group 2 and 3 referred to 50 µg and 300 µg VR-HA respectively with two vaccinations without adjuvant followed by two vaccinations with adjuvant. Group 4, 50 µg (2 birds), and 100 µg (1 bird) VR-HA given two vaccinations without adjuvant. Group 5, 300 µg VR-HA (2 birds) given two vaccinations and one bird given 500 µg VR-HA for three vaccinations. Group 6, inactivated virus vaccine given two vaccinations. Group 7and 8, 100 µg and 500 µg VR-HA given three vaccinations with adjuvant respectively. Group 9 and 10, 100 µg and 500 µg VR-NP given three vaccinations with adjuvant respectively. Group 11, 100 µg blank vector plasmid DNA given three vaccinations with adjuvant.

** For calculation of GMT in those groups with swabs with no detection, the titre of those birds was normally ascribed as an EID_{50} titre of $10^{0.001}$.

3.3.4.3 Comparison of virus recovery between VR-HA, inactivated vaccine and blank vector groups in experiment 2

As illustrated in Table 3.8, there was a highly significant difference in the level of virus shedding in OS (Fisher's exact test, P=0.0001) and CS (Fisher's exact test, P=0.0003) between Group 6 (inactivated vaccine) and Group 11 (blank vector control).

In comparison with Group 11, there was a highly significant decrease in virus shedding in OS (Fisher's exact test, P=0.0006) and CS (Fisher's exact test, P=0.0003) in Group 7 (100 μg VR-HA) whereas there was no significant difference in OS (Fisher's exact test, P=0.29) and CS (Chi-square, P=0.09) in Group 8 (500 μg VR-HA). In comparison with Group 6, there was no significant difference in virus shedding in both OS and CS for Group 7 but there were significant increases in virus shedding in OS and CS for Group 8. Furthermore, there was a highly significant decrease in virus shedding in OS (Chi-square, P=0.0084) and CS (Chi-square, P=0.0013) in combined VR-HA group (Groups 7 and 8) compared to Group 11 whereas there was no significant difference in virus shedding in OS and CS between the combined VR-HA group and Group 6. In terms of virus titre in OS, Group 7 or Group 8 were not statistically significant from each other or from Group 11.

Table 3.8 Level of virus shedding from the VR-HA, VR-NP and control groups post-challenge*.

	Group 6**	Group 7	Group 8	Group7+Group 8	Group 9	Group 10	Group9+Group10	Group 11
OS	2/18(11.1) [a]	3/18(16.7) [a c]	10/18(55.6) [bde]	13/36(36.1) [ace]	13/18(72.2) [bd]	8/18(44.4) [ad]	21/36(58.3) [bde]	14/18(77.8) [bd]
CS	0/18(0) [a]	0/18(0) [ac]	5/18(27.8) [bde]	5/36(13.9) [ace]	0/18(0) [ac]	0/18(0) [ac]	0/36(0) [ac]	10/18(55.6) [bd]

* HA positive numbers/total sample numbers. Percentages are shown in parenthesis.
** Group 6, inactivated virus vaccine given two vaccinations. Group 7and 8, 100 µg and 500 µg VR-HA given three vaccinations with adjuvant respectively. Group 9 and 10, 100 µg and 500 µg VR-NP given three vaccinations with adjuvant respectively. Group 11, 100 µg blank vector plasmid DNA given three vaccinations with adjuvant.
For the row groups with different lowercase superscript letter are significantly different ($P < 0.05$) using Chi-square or Fisher exact tests.

Table 3.9 Proportion of challenged birds that shed virus under each vaccine regime.

VR-HA without adjuvant (Group 4+Group5)		VR-HA with adjuvant (Group2+Group3+Group7+Group8)		VR-NP with adjuvant (Group 9+Group10)		VR with or without adjuvant (Group1+Group11)		IVV(Group6)	
OS	CS	OS	CS	OS	CS	OS	CS	OS	CS
6/6 (100)*	3/6 (50)	10/12 (83.3)	5/12 (41.7)	5/6(83.3)	0/6 (0)	7/7 (100)	6/7 (85.7)	2/5(40)	0/5(0)

* Number of birds shedding virus /total number of birds tested (percentage).

As shown in Table 3.8, there were significant differences in virus shedding from OS (Chi-square, P= 0.035) and CS (Chi-square, P=0.046) between the low dose (Group 7) and high dose (Group 8) VR-HA groups. This was consistent with the result for CS between the low dose VR-HA with lipofectin adjuvant (Group 2) and high dose VR-HA with lipofectin adjuvant (Group 3) in experiment 1. However, there was no statistical significant difference (Mann-Whitney Test, P=1.0) in GMT of virus shedding from OS between Group 7 and Group 8 (Table 3.7).

There was also no significant difference in virus shedding in both OS and CS between the combined groups (Groups 2 and 3) given 4 vaccinations of VR-HA from experiment 1 and those (Groups 7 and 8) given 3 vaccinations of VR-HA in experiment 2. Nor was there any significant difference in the virus GMT between these two combined groups.

3.3.4.4 Comparison of virus recovery between the blank vector control and VR-NP groups in experiment 2

As illustrated in Table 3.8, no virus shedding occurred in the cloaca in both VR-NP groups over 6 consecutive days. Compared to the empty vector group (Group11), there was a 55.6% decrease in virus shedding in CS and a 19.5% decrease in shedding in OS for the combined VR-NP vaccinated group. There was a highly significant decrease in virus shedding in CS but no significant difference for OS between the (combined or single) VR-NP groups and Group11. There was no significant difference in virus shedding in OS and CS between lower dose VR-NP (Group 9) and higher dose VR-NP (Group10) groups. Neither was there any significant difference in the virus GMT in OS between Group 9 and Group 10, and between the combined VR-NP groups and Group 11.

3.3.4.5 Proportion of challenged birds that shed virus under each vaccine regime

As summarized in Table 3.9, by comparison with the empty vector controls, in terms of the proportion of birds that shed virus, there was a decrease of 44% and 85.7% respectively in virus shedding in CS in the adjuvanted VR-HA and the VR-NP group, and a decrease of 16.7% in virus shedding in OS in both groups. However, there was no decrease in the proportion of birds shedding virus in the oropharynx but a decrease of 35.7% in the proportion shedding virus in the cloaca in the non-adjuvanted VR-HA group. In contrast, there was a decrease of 60% in virus shedding in OS and 85.7% in CS in the inactivated vaccine group.

3.4 Discussion

In this chapter, the vaccination of chickens by inoculation of VR1012 plasmid DNAs encoding HA or NP genes from H6N2 AIV was evaluated. Although immunization with VR-HA or VR-NP together with lipofectin adjuvant induced only minimal antibody responses, the vaccinated birds showed some evidence of protection against AIV infection, with a 44% and 85.7% decrease respectively in the number of birds shedding virus via the cloaca but only16.7% reduction in virus shedding via the oropharynx in both groups compared with empty vector vaccinated controls. More interestingly, no virus shedding was observed in CS from both lower dose VR-HA groups and VR-NP groups.

3.4.1 Evaluation of antibody response to DNA vaccines

Both VR-HA vaccines in experiment 1 and experiment 2 and VR-NP vaccines in experiment 2 showed similar results in that they all gave no or poor antibody responses to the H6 virus HA, or NP, even with more sensitive test methods such as ELISA and

immunoblotting assay developed in an attempt to detect marginal antibody levels to HA, in addition to HI test. All DNA vaccinated groups showed rapidly increasing antibody responses to virus challenge as expected, but the post-challenge antibody titres were not statistically different from the blank vector controls. There were statistically significant increases in antibody titre in five out of eight DNA vaccinated groups and the inactivated vaccine group pre- and post-challenge but no significant difference for two blank vector control groups, suggesting that VR-HA, VR-NP or inactivated vaccine vaccination may result in an anamnestic antibody response after challenge. It should be noted that the small group sizes may have resulted in statistical aberrations and this cannot be ruled out. The post-challenge HI titres for VR-NP were significantly lower than those for VR-HA in experiment 2, indicating that the VR-HA vaccine did appear to prime a H6 HA antibody response whereas the VR-NP, as expected, did not. Taken together, the VR-HA DNA vaccines at least provided some evidence of playing a priming role in the antibody response.

The backbone of a DNA vaccine vector carries CpG motifs, which may or may not have an adjuvant effect for DNA vaccines (Doria-Rose and Haigwood 2003). Inoculation of naked plasmid DNA can induce immune responses due to the CpG motif in a regular expression vector (Cantlon *et al.* 2000; Krieg *et al.* 1998a). Although no naive chickens were challenged in the present experiment, in testing post-challenge chicken sera by immunoblotting assay, some blank vector vaccinated chickens gave much stronger non-specific bands than those in VR-HA and VR-NP vaccinated chickens (data not shown). This suggested that the blank vector itself may elicit non-specific immune responses. In fact, the empty VR1012 vaccinated birds also gave strong antibody responses after

challenge, from no antibody pre-challenge to levels equivalent to VR-HA and VR-NP vaccine after challenge and marginally lower than killed vaccine responses. Presumably, the empty vector could non-specifically stimulate immune responses and resulted in enhanced antibody response post virus challenge.

The observation that the HA-expressing DNA vaccines elicited no to very low HI titres in chickens post-vaccination in this study was in an agreement with results using the same VR1012 vector expressing the HA gene of A/turkey/Wisconsin/68(H5N9) virus (Suarez and Schultz-Cherry 2000a). Some studies reported that HA-expressing DNA vaccines elicited no to very low detectable antibody in chickens (Fynan *et al.* 1993a; Kodihalli *et al.* 1997; Kodihalli *et al.* 2000; Robinson *et al.* 1993), mice (Kodihalli *et al.* 1999) and ferrets (Webster *et al.* 1994). But IM injection of plasmid DNA encoding the HA gene of A/HK/156/97(H5N1) in chickens reportedly elicited high HI antibody titres (Chen *et al.* 2001). Chickens administered with a plasmid encoding HA of a LPAI virus (H7N1) by the Medijector device also developed detectable HI titres with the highest geometric titre of $2^{6.8}$ (Cherbonnel *et al.* 2003). The reasons for these differences in efficacy of HA-DNA vaccines are complicated. Many factors, such as DNA expression vector used (Suarez and Schultz-Cherry 2000a), gene inserted (Barry and Johnston 1997; Doria-Rose and Haigwood 2003), animal species (Babiuk *et al.* 2003) or mouse strain (Chen *et al.* 1999a), injected DNA dose (Davis *et al.* 1993b; Doria-Rose and Haigwood 2003; Galvin *et al.* 2000), volume (Dupuis *et al.* 2000), application methods and delivery route (Barry and Johnston 1997; Wang *et al.* 2008b) may all have an effect on the antibody response induced by a DNA vaccine.

3.4.2 Evaluation of the effect of DNA vaccines on virus shedding

AI vaccination has been shown to reduce virus shedding but may not stop it altogether in vaccinated birds (Capua and Marangon 2007a). Robinson *et al.*(1997) demonstrated that post-challenge protection was associated with reduced viral replication in the lungs of mice. Kodihalli *et al.*(2000) showed that the high protection rate induced by either a plasmid encoding HA of A/Turkey/Ireland/83 (H5N8) or a plasmid encoding HA of A/Chick/Victoria/1/85(H7N7) correlated well with a low virus detection rate in tracheal swabs and cloacal swabs as well as low virus titre on day 3 or day 5 following virus challenge of chickens. In the present study, no deaths or clinical signs were expected from an LPAI challenge so reduction of virus shedding in OS and CS was used as an indicator of protection. This reduction in virus shedding was evident in the inactivated vaccine group in these studies.

Two separate trials using VR-HA vaccination with adjuvant showed similar findings. Compared to the inactivated vaccine group, there was no significant difference in the level of virus shedding in OS and CS for the combined adjuvanted VR-HA groups (either the combined group 2 and 3 or the combined group 7 and 8) in both experiments. As compared with the blank vector controls, there was a highly significant decrease in virus shedding in OS and CS in experiment 2, but no significant difference in experiment 1. This indicated that VR-HA vaccination elicited some protective immune response as demonstrated by reduction in virus shedding. However, the reduction rate in virus shedding was greater in the inactivated vaccine group, indicating that this VR-HA vaccine was inefficient at reducing virus shedding following virus challenge in comparison with the inactivated vaccine. Although there was no significant difference in virus titre shed via oropharynx between lower doses and higher doses of the VR-HA

vaccine, the level of virus shedding in CS in the two experiments and in OS in experiment 2 was significantly lower in the lower dose group than in the higher dose group. This suggested that lower doses (either 50 or 100µg) of VR-HA vaccine appeared to induce better protective responses than higher doses (either 300µg or 500µg).

As with the inactivated vaccine, no virus shedding was observed in CS in VR-NP groups or in the lower dose of VR-HA groups. With respect to virus recovery, in comparison with the empty vector group, significant reduction in virus shedding from the VR-NP groups occurred only in CS whereas for the VR-HA groups reduction in virus shedding was apparent in either OS or CS. The reason why VR-HA vaccine had more impact on reducing virus shedding via oropharynx or cloaca whereas VR-NP had more impact on reducing virus shedding via cloaca was not determined from these experiments. This was possibly related to the function of the HA and NP genes. The HA gene plays a more important role in protective immunity than the NP gene (Suarez and Schultz-Cherry 2000b), although NP-mediated CTL response against NP was reportedly vital for limiting the dissemination of the virus, and promoting its clearance in humans (McMichael *et al.* 1983) as well as in mice (Wells *et al.* 1981). However the level of virus shedding via the cloaca after challenge for the empty vector group in this study was low and this may have contributed to no cloacal virus shedding in the adjuvanted VR-NP groups.

As shown in Table 3.6 and Table 3.8, the level of virus shedding via oropharynx or cloaca from VR-HA vaccinated groups without adjuvant (Groups 4 and 5) was not significantly different from the empty vector vaccinated group, indicating that primary immunization induced by two naked VR-DNA vaccinations was not sufficient to generate an effective immune response.

When compared with the unvaccinated controls, HA-DNA vaccinated chickens showed more than 2 log_{10} EID_{50} reduction in virus GMT in tracheal and cloacal swabs collected on day 3 and day 5 after highly pathogenic H5 and H7 AIV challenge and hence conferred good protection (Kodihalli *et al.* 2000). In the current H6N2 LPAI virus study using a high dose challenge, the virus did not seem to replicate well in chickens, resulting in low titre of virus shedding via respiratory and alimentary route in controls but the frequency and titre of virus shedding were variably reduced in either VR-HA or VR-NP vaccinated chickens.

Taken together, using VR-HA or VR-NP constructs at different doses, different numbers of boosters and with or without lipofectin adjuvant did not give good protection against LPAI virus infection in terms of reduction in virus shedding. However, no virus shedding was observed in CS in both lower dose VR-HA groups as well as two VR-NP groups and a reduction in virus shedding in OS in the low dose VR-HA groups. This gives some encouragement for the further development of DNA vaccines against H6N2 AI virus.

3.4.3 Parameters affecting efficacy of DNA vaccines

In early DNA vaccine studies, the dose of the DNA vaccine was considered to be an important factor for the efficacy of DNA vaccines (Robinson *et al.* 1993; Ulmer *et al.* 1994; Ulmer *et al.* 1993). The amount of plasmid DNA used could have as much effect on the strength and character of immune responses as the route of inoculation (Barry and Johnston 1997). A study of pathogenic simian-human immunodeficiency virus (SHIV) in macaques and HIV phase I clinical studies in humans showed that higher doses of plasmid DNA administered gave better antigen-specific immune responses than lower doses (Amara *et al.* 2001; Boyer *et al.* 2000; MacGregor *et al.* 1998). But in another HIV

DNA vaccine study, increasing the vaccine DNA dose did not achieve increased immune responses (Galvin *et al.* 2000). Different levels of DNA vaccine initiated protection were achieved in different experiments in the current study. Lower doses of an HA-expressing DNA vaccine elicited a better reduction in virus shedding, suggesting that the protective immune response of DNA vaccines did not have a direct linear correlation with the dose of inoculated plasmid DNA. This implies that generation of better immune responses to H6 virus cannot be achieved by just increasing the vaccine DNA dose and may require high protein expression by a high-efficiency expression vector or by the use of other boosting agents. This also highlighted that optimization of plasmid DNA dose delivered was necessary for evaluating the efficacy of DNA vaccines. Encouragingly, if lower DNA doses were optimal, the cost of DNA vaccines may potentially be reduced.

In an early HIV experiment, higher amounts of plasmid encoding CTL epitopes of HIV gp160 gave lower CTL responses after single GG immunization (Barry and Johnston 1997). The reason why the lower dose induced better immune response is unknown as the mechanism by which IM injection DNA is introduced into the extracellular spaces of muscle followed by internalization of DNA is as yet fully unidentified (Ulmer *et al.* 1994). Nevertheless, there is some evidence that the level of expression is more crucial for cellular responses. Presumably, large amounts of plasmid might generate too vigorous cellular immune response which perhaps kills off the transfected cells too early to maintain immune stimulation, resulting in attenuated vaccine responses (Barry and Johnston 1997). Moreover, it is believed that in excess of 90% of the DNA by IM injection never enters the cytoplasm and of this 10% less than 1% gets into the nucleus where gene expression occurs (Babiuk *et al.* 2003; Barry and Johnston 1997). Thus, the

possibility that high dose DNA is introduced into extracellular spaces by IM route, potentially leading to a more rapid clearance of DNA, cannot be ruled out.

Lipofectin is generally used for transfection of cells *in vitro*. It has also been used to produce AI HA subtype–specific reference antisera via DNA vaccination of chickens (Lee *et al.* 2006). As illustrated in Table 3.2, in a total of 36 vaccinated birds, 23.8% (5/21) sero-conversion occurred in the lipofectin adjuvant group whereas 13.3% (2/15) seroconversion occurred in the non-adjuvanted group. One chicken developed a low HI titre after the third VR-HA booster given with lipofectin and after a fourth booster given with lipofectin this bird developed a similar HI antibody level to the inactivated vaccine group chickens. This suggested that lipofectin was effective as an adjuvant to improve the level of antibody production in chickens. This is consistent with findings elsewhere of an increase in the percentage of birds that responded to DNA vaccinations given with lipofectin (Suarez and Schultz-Cherry 2000a). Moreover, in experiment 2, 100 µg adjuvanted VR-HA groups showed significant reductions in virus shedding in both OS and CS when compared with the empty vector control group. However, this was not achieved in experiment 1, suggesting that the protocol of three vaccinations with adjuvant in experiment 2 was superior to the protocol with two vaccinations without adjuvant and then with two vaccinations with adjuvant in experiment 1. In comparison with the adjuvanted groups, the IM administration of naked VR-HA plasmid DNA alone two times was not sufficient to induce antibody or to give significant reduction in virus shedding. This supported the use of lipofectin as an adjuvant to provide some enhancement of the immune response induced by the VR-HA or VR-NP DNA vaccines.

DNA vaccinations in other studies required multiple injections before they demonstrated detectable antibody response (Barnett *et al.* 2001; Boyer *et al.* 2000). In this study, one chicken with multiple injections generated a moderate H6 HI antibody titre comparable to that induced by the inactivated vaccine. In two VR-HA experiments, the number of birds showing sero-conversion increased after the third immunization, in contrast to sero-conversion following two vaccinations. This suggests that the number of vaccinations may also play a role in induction of measurable antibody response by DNA vaccines.

3.4.4 LPAI challenge model

A number of studies have evaluated DNA vaccines against HPAI virus. Much less research has been focused on the LPAI model. Because LPAI viruses typically do not cause disease or clinical signs in chickens, assessment of vaccine efficacy is more difficult with an LPAI virus challenge model. A defined challenge model has not been described for LPAI viruses as yet. Cherbonnel *et al.*(2003) and Le Gall-Reculé *et al.*(2007) used a co-infection model with *Mycoplasma gallisepticum* and *Salmonella* spp. in addition to high doses of LPAI virus in an attempt to increase the virus replication and shedding. Prel *et al.*(2007) challenged vaccinated ducks by nasal instillation and eyedrop with a high dose of LPAI virus A/Duck/France/02166/2002(H5N3). In our experiments virus challenge involved a high dose of H6N2 avian influenza virus administered via nasal instillation, eyedrop and oral routes. The results in the blank vector group revealed that the virus was recovered from 100% (7/7) of challenged chickens via OS and 85.7% (6/7) of challenged chickens via CS, indicating that there was sufficient challenge virus for assessment of reduction in virus shedding and that this LPAI virus challenge model was successful in chickens.

In conclusion, two vaccinations with naked plasmid DNA in a VR1012 vector without adjuvant were insufficient to induce an effective immune response against the homologous virus challenge. However, by increasing the number of vaccinations, and with incorporation of lipofectin adjuvant, an enhanced immune response could be induced by VR-HA and VR-NP DNA vaccines. The results described in this chapter provide some encouragement for the further development of a H6 DNA vaccine. In the following chapters we evaluated alternative vectors and adjuvants in attempts to enhance immune response to the H6 DNA vaccines.

Chapter 4

Effect of alternative expression vectors and administration methods on HA-expressing DNA vaccines

4.1 Introduction

In the experiments reported in Chapter 3, a mammalian expression vector VR1012 was used to generate DNA constructs encoding HA or NP genes of A/coot/WA/2727/79 (H6N2) virus. Both VR-HA and VR-NP constructs induced low to no detectable antibody response despite the use of multiple booster injections, lipofectin as adjuvant and a high dose of plasmid DNA. Nevertheless, following homologous virus challenge, vaccination by both VR-HA and VR-NP constructs were able to reduce virus shedding via cloaca and oropharynx to some extent in chickens. Thus, the immunity induced by these DNA vaccines was not sufficiently protective and alternative approaches needed to be investigated.

Selection of an appropriate vector was one major factor that needed to be considered. The DNA expression vector is an important factor affecting the efficacy of DNA vaccines by facilitating expression of the target genes at the transcriptional and post-transcriptional levels (Fattori *et al.* 2002). Characteristics of the nucleotide sequence of the vector itself, e.g. negative regulatory elements, can influence expression levels of the target gene and therefore affect the immunogenicity of DNA vaccines (Montgomery *et al.* 1993). To date, a number of expression vectors have been used in DNA vaccine research. The pCI or pCI-neo vector have been used to generate vaccine plasmid pCI HA or pCI-neo HA which elicited measurable antibody titres in chickens against A/Goose/Guangdong/1/96 (H5N1) or A/turkey/Wisconsin/68 (H5N9) respectively (Chen *et al.* 2001; Suarez and Schultz-Cherry 2000a). The pVAX1 vector was employed to

clone HA, NA and M1 consensus sequences from circulating H5 AIV to generate recombinant constructs, which induced highly cross-reactive cellular immune responses against H5 influenza antigens in mice (Laddy *et al.* 2007).

Another strategy to improve the potency of DNA vaccines is to manipulate the method of plasmid delivery. DNA immunization using conventional needle methodologies, such as IM or ID injections, appeared less successful in non-human primates and humans, however, physical methods, such as GG and electroporation (EP), have been shown to elicit better immune response in large animals and humans (Pertmer *et al.* 1995; Wang *et al.* 2008b). Although IM vaccination has been widely used (Perrie *et al.* 2001), it does not appear particularly efficient at inducing immune responses by DNA vaccination as plasmid DNA administered by IM route was poorly distributed, inefficiently expressed, and rapidly degraded (Otten *et al.* 2000; Ulmer *et al.* 2006; Wang *et al.* 2008b).Gene gun technology, which delivers the DNA coated onto metal carrier beads, usually gold, into epidermal skin cells, was regarded as an effective method for DNA vaccination. But this method is currently impractical for poultry due to the inability to vaccinate large numbers of birds and the high cost of the carrier beads (Fynan *et al.* 1993b; Kodihalli *et al.* 1997; Olsen *et al.* 1997).

Electroporation, also called electropermeabilisation, was first reported for *in vitro* gene transfer in 1982 (Neumann *et al.* 1982). Subsequently, *in vivo* EP has been proven to be an efficient approach for delivering genes into muscle tissue (Mir *et al.* 1999; Rols and Teissie 1998). In comparison with simple muscle injection, EP has been proven to increase gene expression by several orders of magnitude and to significantly reduce inter-individual variability (Fattori *et al.* 2002). This technology has been demonstrated to

increase the magnitude of the humoral and cellular responses induced by DNA vaccines in a range of animal models such as mice (Dupuis *et al.* 2000; Kadowaki *et al.* 2000; Laddy *et al.* 2007; Muthumani *et al.* 2008; Widera *et al.* 2000; Zucchelli *et al.* 2000), rats (Mathiesen 1999; Zucchelli *et al.* 2000), rabbits (Wang *et al.* 2008b; Widera *et al.* 2000; Zucchelli *et al.* 2000), guinea pigs (Widera *et al.* 2000), goats and cattle (Tollefsen *et al.* 2003), sheep (Babiuk *et al.* 2002; Babiuk *et al.* 2007) and non-human primates (Luckay *et al.* 2007). It also allowed a significant reduction in the DNA dose required (Widera *et al.* 2000; Zucchelli *et al.* 2000).

This chapter describes investigations relating to two objectives to try to improve DNA vaccines against H6N2 LPAI virus in chickens.

- To compare three different eukaryotic expression vectors expressing the same HA gene in an attempt to find an improved vector.
- To employ EP to increase DNA uptake by cells and hence expression of the HA gene in chickens.

4.2 Materials and Methods

4.2.1 Subcloning of the H6N2 HA gene into three vectors

The pCI and pCI-neo vector, were kindly provided by Dr David Suarez, Southeast Poultry Research Laboratory, Athens, USA. The pVAX1 vector was kindly provided by Professor Graham Wilcox, School of Veterinary and Biomedical Sciences, Murdoch University, Australia. A schematic showing the genetic maps of the three expression vectors is provided below (Figure 4.1).

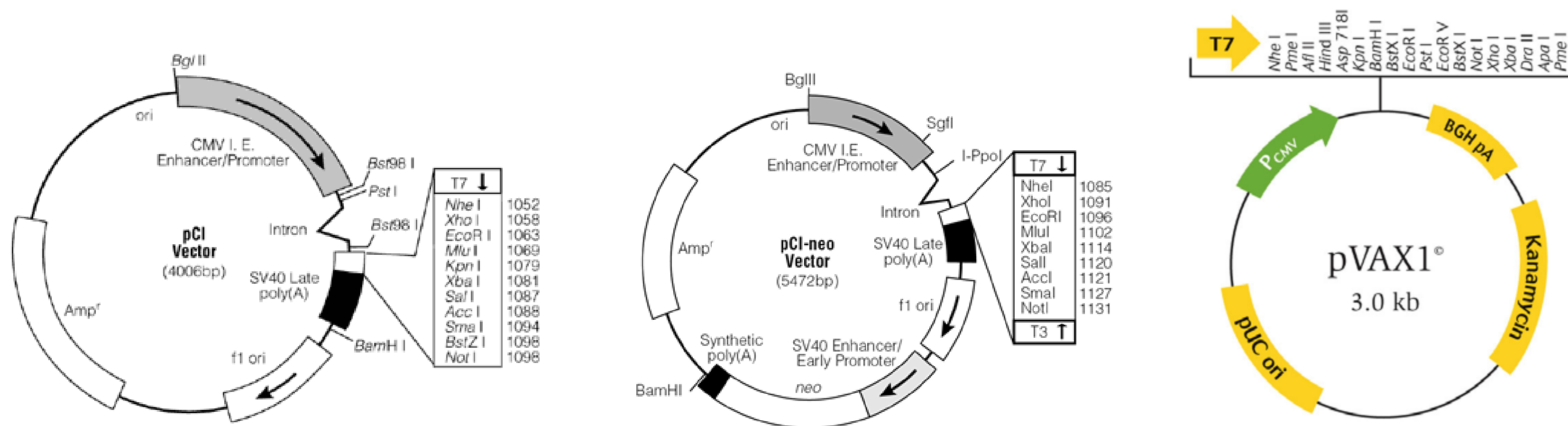

Figure 4.1 Map of the expression vectors used in this study.
The full-length HA fragment was subcloned into the *Eco*RI/*Sma*I multiple cloning site of the vectors pCI, pCI-neo (http://www.promega.com/catalog/catalogproducts.aspx?categoryname=productleaf_8) and the *Eco*RI and *Hin*dIII sites of the pVAX1 (http://tools.invitrogen.com/content/sfs/vectors/pVAX11.pdf), respectively.

The full-length HA gene of A/coot/WA/2727/79 (H6N2) from the pT-HA in Chapter 2 was subcloned into the pCI or pCI-neo vectors at the *Eco*RI and *Xma*I sites to generate pCI-HA and pCI-neo-HA constructs and was similarly subcloned into the pVAX1 vector at the *Eco*RI and *Hin*dIII sites to generate the pVAX-HA construct. Proof-reading PCR was performed as per section 2.2.10.5 using the following primers. The protocol for the subcloning was described in detail in section 3.2.2.

Table 4.1 Primers used for amplification and identification of HA gene from pT-HA.

Primer	Sequence(5'to 3')	Genome	Nt position*
pCI-HAf	ccg**GAATTC**CAAAAGCAGGGGAAAATG**	HA gene	3-20
pCI-HAr	tcc**CCCGGG**TTTCTAATTATATACATATYYTGC	HA gene	1702-1725
HA-pVAXf	Ccc**AAGCTT**CAAAAGCAGGGGAAAATG	HA gene	3-20
HA-pVAXr	ccg**GAATTC**TTTCTAATTATATACATATYYTGC	HA gene	1702-1725
pCIf	TCCACTTTGCCTTTCTCTCCAC	pCI vector	966-987
pCIr	TCATCAATGTATCTTATCATG	pCI vector	1115-1136
pVAXf	TGGGAGGTCTATATAAGC	pVAX vector	596-613
pVAXr	AGGGGCAAACAACAGATG	pVAX vector	849-867

* The nucleotide (nt) numbering system of was adopted as per the HA gene of A/coot/WA/2727/79 (H6N2) virus.
**Restriction endonuclease sequence is in bold.

4.2.1.1 Purification of PCR products

PCR products were purified using a Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. In brief, the PCR product was dissolved in 5 x volume Buffer PB. After mixing, the mixture was added into a spin column followed by centrifugation at 13,000 g for 60 sec at room temperature. After discarding the flow-through, 750 µL buffer PE was added followed by two centrifugations at 13,000 g for 60 sec. The column was then placed into a 1.5 mL Eppendorf tube and 30 µL dH_2O was added. After standing for 1 min at room temperature DNA was eluted by centrifugation at 13,000 g for 60 sec and stored at -20°C.

4.2.1.2 Restriction endonuclease reactions

Restriction endonuclease reactions were performed as described in section 3.2.1.1 but different buffers and enzymes were used. For cloning into the pCI or pCI-neo vectors, 4 µL of 10 x buffer B (Promega), 0.4 µL acetylated BSA (10 µg/µL), 2 µg plasmid DNA (pCI-neo, pCI or purified PCR product), 1 µL *Eco*RI (12 U/µL, Promega), 2.0 µL *Xma*I (5 U/µL, Promega) were mixed with dH_2O made up to 40 µL. The reaction mix was incubated at 37°C for 2-4 hr.

For cloning into the pVAX1 vector, 4 µL of 10 x buffer B (Promega), 0.4 µL acetylated BSA (10 µg/µL), 2 µg plasmid DNA (pVAX1 or purified PCR product), 1 µL *Eco*RI (12 U/µL, Promega) , 1.0 µL *Hin*dIII (10 U/µL, Promega) were mixed with sterile, dH_2O made up to 40 µL. The reaction mix was incubated at 37°C for 2-4 hr.

For the pVAX-HA identification, 2 µL of 10 x NEBuffer (New England Biolabs Inc., Hertfordshire, UK), 0.2 µL acetylated BSA (10 µg/µL), 1 µg pVAX-HA DNA, 0.5 µL *Eco*R I (12 U/µL, Promega), 0.6 µL *Apa*LI (10 U/µL, NEB) were mixed with dH_2O up to 20 µL. The reaction mix was incubated at 37°C for 2 hr.

4.2.1.3 Ligation and transformation

Ligation and transformation were conducted as described in 3.2.2.2. Plasmid DNA containing the recombinant constructs was prepared as described in section 3.2.3.

4.2.2 Transient expression of recombinant plasmids in eukaryotic cells

As described in section 3.2.4.1, Cos-7 cells were prepared without antibiotics until forming 80-90% confluency in a 6-well plate. The cells were transfected transiently using

FuGENE® HD Transfection Reagent (Roche Diagnostics Australia Pty. Ltd., NSW, Australia) following the manufacturer's protocol. Briefly, 5 μg plasmid DNA was added into 100 μL opti-MEM (Gibco BRL) in a 1.5 mL Eppendorf tube. Then 15 μL transfection reagents were added into the DNA solution without contact with the tube wall followed by vortexing for 1-2 sec and incubating at room temperature for 15 min. The DNA mixture was dispensed into 6-well plates in a drop-wise manner then the plate was incubated at 37°C for 48 hr.

The expression of recombinant constructs in Cos-7 cells was detected by WB as per section 3.2.5. After the removal of the media, 90 μL non-reducing loading buffer was added into each well. The lysate was boiled for 10 min prior to loading into the electrophoresis gel. After WB, the nitrocellulose membrane was scanned in the ProXPRESS 2D Proteomic Imaging System (PerkinElmer, CT, USA). IFT was performed as per section 2.2.5 using a microscope (Olympus BX51, Tokyo, Japan).

4.2.3 Experimental design of the vaccination and challenge study

Three-week old Hy-Line layer pullets (Altona Hatchery Pty. Ltd.) were raised in a free-range pen. DNA vaccine formulations were conducted as described in section 3.2.9. The immunization regime is shown in Table 4.2 according to the procedure described previously (Lee *et al.* 2003). Each group of 5 birds received two separate injections in the breast muscle of 0.2 mL plasmid DNA diluted in PBS. Birds were subsequently given booster vaccinations twice at 4-week intervals using the same DNA vaccine. The vaccinated birds were bled every two weeks to determine HI antibody titres.

Each chicken received 1 mL ($10^{6.25}$ EID_{50}/0.1 mL) of the challenge virus 6-7 weeks after the third vaccination as described in section 2.2.8. Swabs were collected daily for virus isolation as described in section 2.2.9.

Table 4.2 Immunization procedure for pCI-HA, pCI-neo-HA and pVax-HA DNA vaccine in chickens.

Group	Species	Number of birds	Vaccine	Dose	Route*	Adjuvant	Number of immunization
1	Hy-line	5	pCI-HA	50	IM	No	3
2	Hy-line	5	pCI-HA	100	IM	No	3
3	Hy-line	5	pCI-HA	300	IM	No	3
4	Hy-line	5	pCI-neo-HA	50	IM	No	3
5	Hy-line	5	pCI-neo-HA	100	IM	No	3
6	Hy-line	5	pCI-neo-HA	300	IM	No	3
7	Hy-line	5	pVAX-HA	50	IM	No	3
9	Hy-line	5	pVAX-HA	100	IM	No	3
9	Hy-line	5	pVAX-HA	300	IM	No	3
10	Hy-line	4	pCI-HA	100	IM	Lipofectin	3
11	Hy-line	4	pCI-neo-HA	100	IM	Lipofectin	3
12	Hy-line	5	Inactivated virus	1mL	IM	No	2
13	Hy-line	4	PBS	1mL	IM	No	3
14	White Rock	8	pCI-HA	100	IM	No	2
15	White Rock	8	pCI-HA	100	EP	No	2

* IM refers to intramuscular delivery and EP represents electroporation delivery.

In a separate experiment, 3-week-old SPF White Rock chickens, from Harbin Veterinary Research Institute, China, were housed in HEPA-filtered isolators. Each group of 8 birds was inoculated twice with pCI-HA at a 3-week interval via IM route alone or by EP according to the manufacturer's instructions. Briefly, following IM injection of plasmid DNA in a 200µL volume at two sites in the leg, a pair of surface plate electrodes was clipped to the leg at the DNA injection sites. The EP parameters used were as follows: voltage- 220V, discharge magnitude - 60ms and frequency -50HZ, using an electric pulse

generator (WJ-2002 Electrode Device for simultaneous injection of DNA and electrotransfer, Scientz Biotechnology Co., Ltd., Ningbo, China). Some fasciculation of the chicken's legs occurred with electroporation and the procedure was repeated using a pulse of the opposite polarity.

4.3 Results

4.3.1 Identification of recombinant constructs in three vectors

The three constructs produced fragments of the expected sizes following restriction endonuclease digestions as shown in Figure 4.2. As expected from the restriction sites of DNA constructs, the pCI-HA gave 1.7 kb and 4.0 kb fragments following *Eco*RI and *Xma*I digestion and 3.72 kb and 1.98 kb following *Eco*RI and *Bam*HI digestion. The pCI-neo-HA gave 1.7 kb and 5.4 kb fragments following *Eco*RI and *Xma*I digestion and 3.1 kb and 4.0 kb following *Eco*RI and *Bam*HI digestions. The pVAX-HA gave 1.7 kb and 3.0 kb fragments following *Eco*RI and *Hin*dIII digestion, 1.9 kb and 2.8 kb fragments following *Eco*RI and *Apa*LI digestion, and 4.7 kb following *Eco*RI and *Xma*I digestion or *Eco*RI and *Bam*HI digestion. These three constructs were also further characterized for the fidelity of the HA gene and insertional direction using sequencing and alignment by ClustalW as expected.

4.3.2 *In vitro* expression of the three HA-expressing constructs

The expression of the three HA-expressing constructs in Cos-7 cells by WB displayed an approximately 84 kD band (Figure 4.3, left). The pCI-HA gave the strongest band followed by the pCI-neo-HA. The pVAX-HA band was barely detectable. With an increase to the amount of plasmid DNA for transfection, the band intensity from both the

pCI-neo-HA and pVAX-HA constructs became denser (Figure 4.3, right). The ratio of FuGENE® HD Transfection Reagent (µl): pVAX-HA DNA of 10:5 gave the best transfection result.

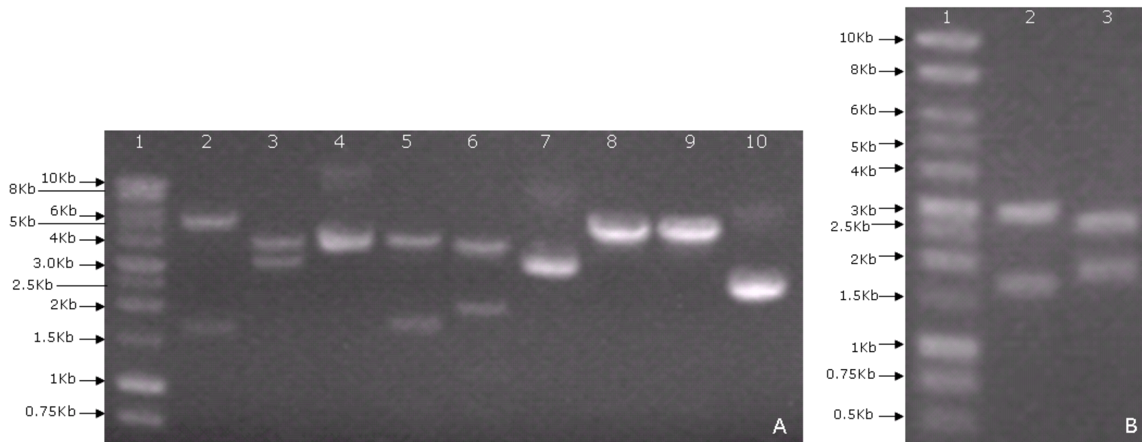

Figure 4.2 Electrophoresis gels showing restriction enzyme digestion patterns of DNA vaccine constructs.
A Lane 1, 1kb DNA ladder (Promega); lane 2, 3 and 4, pCI neo-HA double digested with *Eco*RI and *Xma*I, *Eco*RI and *Bam*HI, and undigested pCI-neo-HA plasmid DNA; lane 5, 6 and 7, pCI-HA double digested with *Eco*RI and *Xma*I, *Eco*RI and *Bam*HI , and undigested pCI-HA; lane 8, 9 and 10, pVAX-HA double digested with *Eco*RI and *Xma*I, *Eco*RI and *Bam*HI, and undigested pVAX-HA. **B** Lane 1, 1kb DNA ladder, lane 2, pVAX-HA digested with *Eco*RI and *Hin*dIII, lane 3, pVAX-HA digested with *Eco*RI and *Apa*LI.

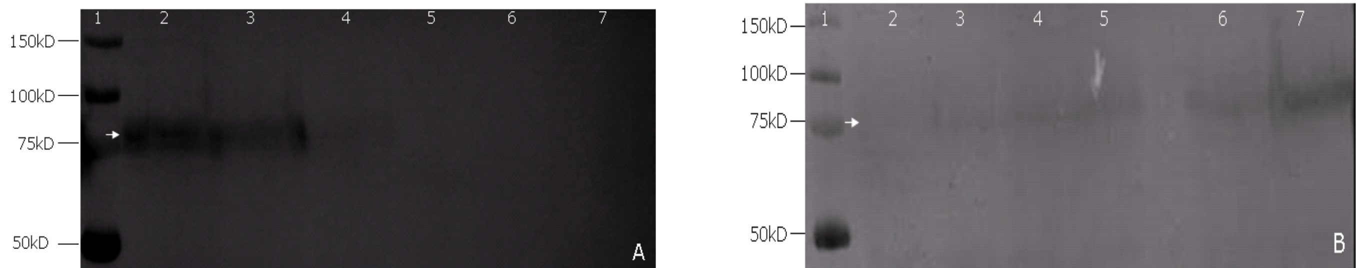

Figure 4.3 Transient expression of three DNA vaccine constructs by Western blotting.
A Lane 1, Marker (Bio-Rad); lane 2 , pCI-HA; lane 3, pCI-neo-HA; lane 4, pVAX-HA; lane 5, pCI; lane 6, pCI-neo; lane 7, pVAX1. **B** Lane 1, Marker, lane 2-5, FuGENE® HD Transfection reagent (µL): pVAX-HA DNA (µg) ratios: 6/3, 10/5, 15/5, 20/5; lane 6-7, FuGENE® HD Transfection reagent (µL):pCI-neo-HA DNA (µg) ratios: 6/3, 10/5.

Cos-7 cells transfected with pCI-HA or pCI-neo-HA displayed bright fluorescence in the cytoplasm and cell membrane using anti-H6 serum and the IFT (Figure 4.4), however, the pVAX-HA transfected cells showed only limited fluorescence.

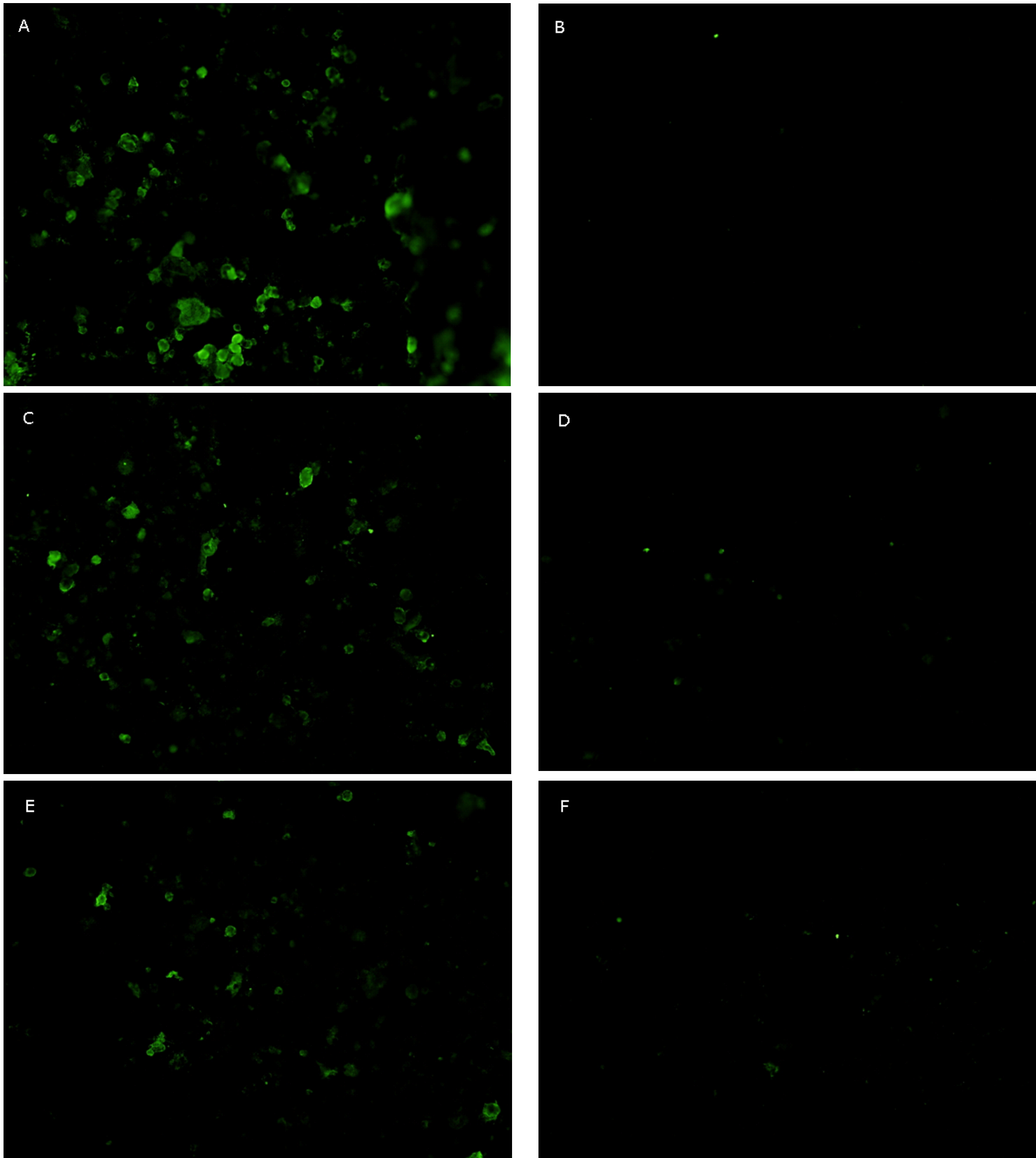

Figure 4.4 Fluorescence occurring in three H6 HA-expressing DNA vaccine constructs expressed in Cos-7 cells.
A, pCI-HA; B, pCI; C, pCI-neo-HA; D, ,pCI-neo; E, pVAX-HA; F, pVAX1 vector. (x10)

4.3.3 Antibody responses in intramuscularly vaccinated chickens

4.3.3.1 Comparison of antibody responses induced by three non-adjuvant DNA vaccines

No HI titre was detected in any vaccinated chickens 6 weeks after the third vaccination. However, at 7 days post virus challenge the chickens showed a range of HI titres (Table 4.3). Except for the inactivated vaccine group, there was a significant increase in the GMT of HI antibody between pre- and post-challenge in all HA DNA vaccinated groups and the naïve control group using the paired-sample T test. There was no significant difference in the GMT of HI antibody post challenge between the naïve control group and each of the DNA vaccine groups (single or combined), whereas, the post-challenge GMT for the inactivated vaccine group was significantly higher than each of the non-adjuvanted DNA vaccine groups. There was also a significantly higher (ANOVA, P=0.000) HI GMT post challenge in the inactivated vaccine group compared with the naïve control group.

There was no significant difference in the GMT of HI antibody post challenge for different doses in each vaccine pCI-HA, pCI-neo-HA or pVAX-HA (ANOVA, P=0.073, 0.41 and 0.086 respectively). Neither was there significant difference in the GMT of HI antibody post challenge between the three vaccines (ANOVA, P=0.53).

Table 4.3 Antibody response prior to challenge (PRC) and post challenge (POC) for naive controls, inactivated vaccine (IVV) and different DNA vaccine groups (pCI-HA, pCI-HA-neo and pVAX-HA) at the doses indicated (in µg).

	Naive control	IVV	pCI-HA				pCI-HA-neo				pVAX-HA		
			50	100	300	100*	50	100	300	100*	50	100	300
PRC	0** ^{Aa}	8.6±1.1 $^{A\,b}$	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}	0 ^{Aa}
POC	4.5±0.6^{Ba}	9.8±0.5 ^{Ab}	3.2±1.3 ^{Bac}	5.2±1.6 ^{Bac}	5.2±1.3 ^{Bac}	8.3±1.3 ^{Bdf}	4.4±2.6 ^{Bac}	5.4±1.1 ^{Bac}	6.2±2.2 ^{Bacf}	4.8±1.0 ^{Bae}	4.9±2.1 ^{Bac}	3.6±1.1 ^{Bac}	6.4±2.1 ^{Bacf}

* Adjuvant group.
** HI titre of each group shown as GMT(log_2) ± standard deviation (SD).
Within the column the different uppercase superscript letter indicates statistical differences ($P < 0.05$) using Paired-sample T test. For the row groups with different lowercase superscript letter are significantly different ($P < 0.05$) using ANOVA or Mann-Whitney test.

4.3.3.2 Effect of adjuvant on antibody response

As shown in Table 4.3, the HI titre in 100 µg pCI-HA vaccinated chickens ranged from 2^3 to 2^7 with a GMT of $2^{5.2}$ after virus challenge. The HI titre in 100 µg adjuvanted pCI-HA vaccinated chickens ranged from 2^7 to 2^{10} with a GMT titre of $2^{8.3}$. There was a significant difference (ANOVA, P=0.019) in the GMT of HI antibody between non-adjuvanted and adjuvanted pCI-HA groups. There was also a statistically significant difference in GMT post challenge between the 100µg pCI-HA adjuvanted group and the naive control group (ANOVA, P=0.002), the 100µg pCI-HA adjuvanted group and the inactivated vaccine control group (ANOVA, P=0.036).

The HI titre in 100 µg pCI-neo-HA vaccinated chickens ranged from 2^4 to 2^7 with a GMT of $2^{5.4}$ after virus challenge whereas the HI titre in 100 µg adjuvanted pCI-neo-HA vaccinated chickens ranged from 2^4 to 2^6 with a GMT of $2^{4.8}$. There were no significant differences (ANOVA P=0.39) in the GMT of HI antibody between the non-adjuvant and adjuvant pCI-neo-HA groups. Neither was there a statistically significant difference (ANOVA, P=0.67) in GMT post challenge between 100µg pCI-neo-HA adjuvanted group and the naive control group.

4.3.3.3 Effect of poultry breed on antibody response

The HI antibody GMT induced by pCI-HA vaccines in White Rock SPF chickens are shown in Table 4.4. Two out of eight SPF chickens gave weak, transient HI titres following two vaccinations with pCI-HA, but there was no significant difference (Mann-Whitney Test, P=0.24) in the GMT of HI antibody at two weeks after the second

immunization between White Rock (SPF) and Hy-Line (commercial) chickens intramuscularly vaccinated with 100µg pCI-HA.

Table 4.4 Comparison of HI antibody GMT in SPF chickens immunized with 100 µg pCI-HA vaccine administered by intramuscular injection (IM) or electroporation (EP).

Group	Route	HI titre (weeks post vaccination)							
		1	2	3*	4	5	6	7	8
1	IM	0	0	0	0	0.1±0.2	0.06±0.2	0	0
2	EP	0	0	0.3±0.7	0.6±1.8	1.3±2.1	1.1±2.1	0.8±1.9	0.8±1.9

* The second vaccination. HI titre for each group showed as GMT (log_2) ± SD.

4.3.3.4 Antibody response in chickens vaccinated using electroporation

The antibody response curve induced by pCI-HA administered with EP is shown in Figure 4.5. One out of eight birds showed a low HI titre following the first vaccination. Two weeks following the booster, four out of eight birds seroconverted and the highest HI titre reached 2^6. However, there was no significant difference (Mann-Whitney Test, P=0.182) in the GMT of HI antibody between the IM and EP groups at 5 weeks post-vaccination (Table 4.4).

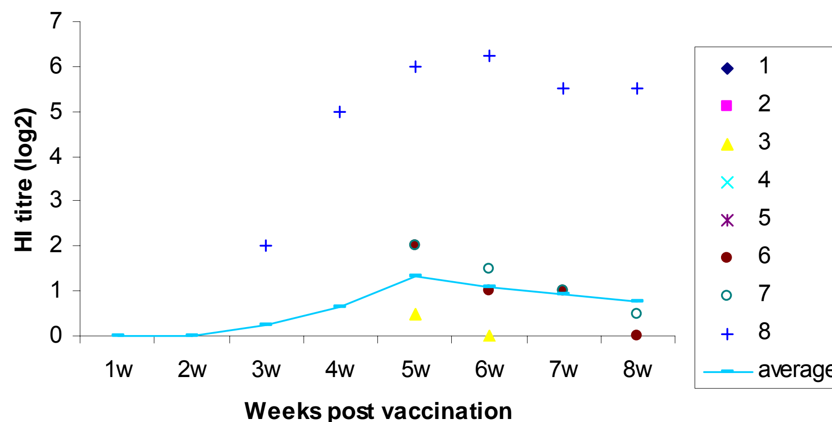

Figure 4.5 Temporal anti-H6 HI responses in chickens immunized with 100µg pCI-HA by EP.
The curve represents the HI antibody GMT of eight birds. Each icon number refers to one bird.

Table 4.5 Virus shedding post challenge in oropharyngeal (OS) and cloacal swabs (CS) in unvaccinated chickens and chickens vaccinated with inactivated vaccine (IVV) or different DNA vaccine groups (pCI-HA, pCI-neo-HA and pVAX-HA) at the doses (µg) indicated.

	Naive control	IVV	pCI-HA					pCI-neo-HA					pVAX-HA			
			50	100	300	Sub-total	100**	50	100	300	Sub-total	100**	50	100	300	Sub-total
OS	13/28 (46.4)*[a]	0/35 [bc]	15/35 (42.9) [ade]	14/35 (40.0) [ade]	25/35 (71.4) [bcf]	54/105 (51.4)	9/28 (32.1) [ad]	19/35 (54.3) [ac]	17/35 (48.6) [ad]	12/35 (34.3) [ad]	48/105 (45.7)	9/28 (32.1) [ad]	16/28 (57.1) [ac]	19/35 (54.3) [ac]	15/35 (42.9) [ad]	50/98 (51.0)
CS	9/28 (32.1)[a]	4/35 (11.4) [ac]	3/35 (8.57) [bc]	0/35[bc]	3/35 (8.57) [bc]	9/105 (8.57)	0/28[bc]	3/35 (8.57) [bce]	0/35 [bc]	7/35 (20.0) [aef]	10/105 (9.52)	1/28 (3.57) [bcf]	0/28 [bc]	4/35 (11.4) [ac]	6/35 (17.1)[a]	10/98 (10.2)

* No. of bird swabs positive for virus /total number of swabs tested from birds in the group (percentage), ** Adjuvant group.
For the rows groups, statistical analysis is performed only in each of three HA-expressing construct groups, naïve control and inactivated vaccine group. Groups with different lowercase superscript letter in the row are significantly different ($P < 0.05$) using Chi-square or Fisher's exact test.

4.3.3.5 Virus shedding in vaccinated chickens following virus challenge

The level of virus shedding in vaccinated and control groups after challenge with H6N2 virus was summarized in Table 4.5.There was a highly significant difference (Fisher's exact test, P=0.000) in the level of virus shedding in OS but no significant difference (Fisher's exact test, P=0.062) in CS between the inactivated virus vaccine (IVV) and the naïve control groups.

There was no significant difference in virus shedding in OS between the naïve control group and any of the HA-expressing DNA vaccine groups except for the 300µg pCI-HA group (Chi-square, P= 0.043). However, there were significant differences in the level of virus shedding in CS between the naïve control group and the groups vaccinated with 50, 100 and 300 µg pCI-HA; 50 and100 µg pCI-neo-HA; or 50 µg pVAX-HA (P< 0.05). For adjuvanted groups, there were no significant differences in virus shedding in OS but significant differences in CS between the naïve control group and either the adjuvanted 100 µg pCI-HA (Fisher's exact test, P=0.0018) or 100 µg pCI-neo-HA groups (Fisher's exact test, P=0.012).

In comparison with the IVV group, there were no significant differences in virus shedding in CS in any of the HA-expressing DNA vaccine groups, but there were significant differences in virus shedding in OS compared with the 50 µg, 100 µg pCI-HA, 100µg, 300 µg pCI-neo-HA, 300 µg pVAX-HA, adjuvanted 100 µg pCI-HA and adjuvanted pCI-neo-HA groups (P< 0.05).

There were significant reduction in virus shedding via the oropharynx in chickens between the 50 µg and 300 µg (Chi-square, P= 0.016), 100 µg and 300 µg (Chi-square,

P= 0.008) of the pCI-HA vaccine. Significant differences also occurred in cloacal shedding between 100 µg and 300 µg doses (Fisher's exact test, P=0.029) of the HA-pCI-neo vaccine and between 50 µg and 300 µg doses (Fisher's exact test, P=0.011) of pVAX-HA. The results from all these groups suggested that lower doses of these vaccines were more effective in reducing the level of virus shedding than the higher dose.

Additionally, there was no significant difference in virus shedding between 100 µg non-adjuvanted and adjuvanted pCI-HA groups (Chi-square, P= 0.52 in OS, Fisher's exact test, P=1.0 in CS) or pCI-neo-HA groups (Chi-square, P= 0.19 in OS, Fisher's exact test, P=0.44 in CS). There was no significant difference in virus shedding in OS or CS between the combined pCI-HA, pCI-neo-HA and pVAX-HA groups.

4.4 Discussion

Direct IM injection of plasmid DNA has been used extensively for immunization against a range of antigens but its mechanism is not clearly understood. Different DNA expression vectors that contain different combinations of natural and/or synthetic promoters, introns and transcriptional termination signals have been compared in an attempt to increase the transcriptional level in transduced muscle and hence improve the DNA expression (Fattori *et al.* 2002). The first objective in investigations reported in this chapter was evaluation of three different plasmid vectors for induction of immune responses to H6N2 HA. Antibody titres in vaccinated chickens have been shown to correlate well with survivability after HPAI virus challenge (Suarez and Schultz-Cherry 2000a). Alternatively, a decrease in viral shedding in the trachea and cloaca has also been identified as an excellent marker for determining protection from AIV challenge after vaccination (Crawford *et al.* 1999). Although the three DNA plasmid constructs did not

elicit measurable H6 HA antibody responses in vaccinated Hy-Line chickens, DNA vaccination significantly reduced virus shedding in CS post-virus challenge. This indicated that the three vaccines had induced some level of immunity in the vaccinated chickens. Moreover, in the 100 μg pCI-HA adjuvanted group the post-challenge HI antibody titres were significantly higher than post-challenge HI titres for the naïve control group, indicating an anamnestic antibody response after priming induced by the DNA vaccination. However, virus shedding was not significantly reduced in OS, indicating that these three DNA constructs were not effective in protecting the oropharynx against homologous H6N2 AIV infection.

Although there was no significant reduction in virus shedding in OS for the different doses of each vaccine, the lower dose groups (50 or 100 μg) appeared to be more effective at reducing virus shedding in CS than the higher dose group (300 μg). This was consistent with the results reported in Chapter 3 with the VR-HA construct. It has been previously reported that expression of injected plasmid DNA was not directly proportional to delivery dose and that optimization of DNA immunization is necessary (Davis *et al.* 1993b).

The *in vitro* expression studies showed that pCI-HA gave the highest level of protein expression in Cos-7 cells among the three constructs. Although there was no statistical difference in reducing virus shedding in OS and CS between the three vaccine groups *in vivo*, both the 50 and 100 μg pCI-HA groups tended to have lower levels of virus shedding than the other two constructs (Table 4.5). Moreover, the adjuvanted pCI-HA group showed better priming immune responses, resulting in higher post-challenge antibody titres, than the adjuvanted pCI-neo-HA group. Thus, both the *in vitro* and *in*

vivo expression studies indicated that the pCI-HA construct was more effective as a vaccine than the pCI-neo-HA or pVAX-HA constructs.

Although numerous vectors have been used in genetic immunization, little has been done to investigate the effect of different vectors on the generation of immune responses to DNA vaccines. In a study with H5 HA-expressing DNA vaccines using four different promoters in five different plasmids (pSI, pCI-neo, VR1012, pCAGGS.MCS and pRC/RSV), the pCI-neo HA construct with the CMV-IE promoter afforded the best response on the basis of antibody titre and protection post-virus challenge (Suarez and Schultz-Cherry 2000a). In comparison with the pCI, the pCI-neo was designed to contain a β-globin/IgG chimeric intron located downstream of the enhancer/promoter region, which was considered to further increase gene expression (Promega 2007). However, results from our study did not demonstrate that the pCI-neo vector was more effective than the pCI vector. Possibly, due to low efficiency of gene uptake and/or expression for both constructs via IM route, the larger size of the pCI-neo-HA construct may have been more unfavorable for myofibre cell uptake or expression. The pVAX1 plasmid lacks an intron in its backbone in contrast to the pCI and pCI-neo plasmids. The addition of an intron to the plasmid is believed to cause enhanced protein expression (Manoj *et al.* 2004). Intron A acted as an enhancer of the CMV-IE promoter and positively regulated expression from the CMV-IE promoter in transformed mammalian cells (Chapman *et al.* 1991). Introns can also give rise to small regulatory mRNA molecules that regulate gene expression levels (Lin *et al.* 2006). Thus, absence of an intron in the pVAX 1 vector may have contributed to its lower gene expression level.

Spatial–temporal patterns of gene expression in mouse skeletal muscle revealed that the majority of DNA was degraded before entering muscle cells and an average of about 6% (up to 15%) of the myofibre cells surrounding the injection site took up and expressed DNA (Doh *et al.* 1997). Thus, a suggested approach to enhance the potency of DNA vaccines was to increase the proportion of transduced myofibre cells within a given muscle. *In vivo* electroporation has been demonstrated to increase both the number of muscle fibres taking up plasmid DNA and the copy number of plasmids introduced into the cells (Aihara and Miyazaki 1998). Comparison of IM and EP to induce immune responses to DNA vaccination was the second objective of the investigations reported in this chapter.

Although GG is one of the most popular methods for DNA vaccine delivery, EP was chosen in this study as it appeared to be most suitable. Some studies have demonstrated that EP based DNA intramuscular delivery resulted in higher and more persistent levels of protein expression compared to GG or IM vaccination (Best *et al.* 2009; Bloquel *et al.* 2006). Another study showed in an anti-tumor model in transgenic mice that GG seemed to be less effective than EP in controlling the incidence and the growth of spontaneous tumors albeit the overall antigen specific immune responses between GG and EP were very similar (Smorlesi *et al.* 2006). In addition, compared to GG, EP may be more practical for routine use as the preparation of DNA coated particle beforehand and pre-treatment (depilation) at the injection site are unnecessary.

In the experiments with VR-HA via IM injection reported in Chapter 3, no or weak antibody responses were induced before virus challenge, and similar findings were shown with DNA vaccines using the three alternative HA-expressing constructs via IM injection.

However, in contrast to the IM vaccination groups, administration of 100 μg pCI-HA via EP resulted in 50% percent of vaccinated chickens showing evidence of sero-conversion, with HI titres in some birds reaching 2^6, although the GMT of HI antibody for the EP group was not significantly different from the group receiving this vaccine dose by IM injection. This is consistent with the results of studies in which a codon optimized H5 HA DNA vaccine administered via EP induced earlier and higher antibody titres in rabbits than via IM injection alone (Wang *et al.* 2008b). The results reported here suggest that EP may also be effective in enhancing the efficacy of DNA vaccination in chickens.

Another study showed that a HA-expressing DNA construct induced high, intermediate and low levels of ELISA antibody in BALB/c (H-2^d), C3H (H-2^k), and B10 (H-2^b) mice respectively and the HA-DNA conferred significant protection only in BALB/c mice (Chen *et al.* 1999b). Similarly, in a study with DNA plasmids encoding for five *Plasmodium falciparum* proteins, mouse strain affected antibody response (Sedegah *et al.* 2004). Such variability between different strains of mice highlighted the importance of genetic background on immune response. In the current studies very low or no HI antibody titres were induced by the pCI-HA vaccine given IM in either White Rock or Hy-Line chickens. It appeared that there was no significant difference between the chicken breeds in the uptake and expression of the pCI-HA plasmid.

Like the non-adjuvanted vaccine groups, the 100 μg pCI-HA and pCI-HA-neo adjuvanted groups showed significantly reduced virus shedding in the cloaca than naïve controls, although this reduction did not occur with virus shedding in the oropharynx. Although there was no significant difference in virus shedding in the oropharynx and cloaca between the non-adjuvanted and adjuvanted groups, there was a significantly

higher GMT of HI antibody in the 100μg pCI-HA adjuvanted group post challenge, indicating more effective priming in the adjuvanted vaccine group and that lipofectin adjuvant played a role in improving the immune response for the pCI-HA vaccine.

Although the three DNA constructs tested in this phase of the study did not inhibit replication of the challenge virus in the oropharynx after conjunctival, nasal and oral challenge, they were able to substantially reduce virus shedding from the cloaca. No challenge virus could be recovered from about 90% of CS from the birds receiving one of the three DNA constructs, whereas, challenge virus was isolated from about 32% of CS from the naïve control group (Table 4.5). Because cloacal shedding of infectious virus and subsequent contamination of litter or water is a major source of AI virus in the environment (Condobery and Slemons 1992; Crawford *et al.* 1998; Wood *et al.* 1994), DNA vaccines based on these plasmid-HA constructs should be effective at reducing transmission of AIV in the field even if no measurable antibody response was induced.

Since HA plays a more important role in inducing antibody response to avian influenza than NP and the aim of this thesis was to addresses the low immunigenicity of plasmid DNA, this chapter and subsequent chapters only focused on the HA-expressing plasmid. This Chapter reported the results of experiments using three alternative eukaryotic vectors encoding the same HA gene of a H6N2 virus. The investigations showed that the pCI-HA construct was better than the other two constructs in terms of antibody response and reduction in the level of virus shedding, although all three vaccines were not very effective at inducing a protective immune response. However, it was encouraging to note that the use of lipofectin as an adjuvant and EP as the delivery regime were both shown to enhance the immune responses induced by the pCI-HA construct. This indicated that

further strategies were now needed to improve the potency of DNA vaccines for the protection of chickens against the H6N2 AI virus.

Chapter 5

Enhanced efficacy of an HA-expressing DNA vaccine using Kozak sequence and an alternative pCAGGGS vector

5.1 Introduction

Although immunogenicity and protective efficacy of DNA vaccines have been shown in numerous model systems, one of the problems hindering DNA vaccine development has been low immunogenicity (Abdulhaqq and Weiner 2008). The development and evaluation of H6-HA DNA vaccines using four different DNA expression vectors, VR1012, pCI, pCI-neo and pVAX1, have been described in the two previous chapters. All these constructs containing a CMV promoter induced weak or no H6 antibody response in vaccinated chickens, even when co-administered with Lipofectin as adjuvant. However, some protective efficacy in terms of reduction in virus shedding after virus challenge was demonstrated. Therefore, other parameters affecting the expression of the HA from the DNA construct needed to be explored.

The appropriate design of the plasmid is crucial to high level expression of its encoded antigen and subsequent immune response induced by that antigen. Among many factors affecting the level of antigen expression in eukaryotic expression vectors, the promoter is regarded as an important variable (Davis *et al.* 1993b). Although the CMV promoter appeared to be the most efficient in a number of studies (Manoj *et al.* 2004), poor immune responses resulted from DNA constructs using CMV promoters in the studies in chickens reported in Chapters 3 and 4. Thus it was decided to use another expression vector containing a different promoter. The vector pCAGGS, containing the chicken β-actin promoter, has been shown to be effective in DNA vaccines for Nipah virus (Wang *et al.* 2008c), Rift Valley fever virus (Wang *et al.* 2007), H5 AIV (Jiang *et al.* 2007), HIV

(Bu *et al.* 2003), hepatitis B virus (Oka *et al.* 2001), influenza (Chen *et al.* 1998b), Japanese cedar pollinosis (Masuda 2005), and diabetes (Shigihara *et al.* 2005).

A Kozak sequence is regarded as the consensus sequence for initiation of translation in vertebrates (Kozak 1987a, b). It functions by slowing down the rate of scanning by the ribosome and increasing the chance of ribosome recognising the start of translation at the AUG start codon. Insertion of a Kozak sequence into a gene of interest or into an expression vector has been used to develop DNA vaccines against Chikungunya virus (Muthumani *et al.* 2008), HIV (Kumar *et al.* 2006), duck hepatitis B (Xu *et al.* 2005), enterotoxigenic *E coli* (ETEC) infection (Melkebeek *et al.* 2007), and minigene DNA vaccines (An *et al.* 2000).

In this study, we incorporated a Kozak sequence upstream of the initiation codon of the HA gene and then cloned this modified HA gene into the expression vector pCAGGS, resulting in a pCAG-HAk construct. This DNA vaccine construct was subsequently evaluated *in vitro* and by vaccination and challenge studies in chickens.

5.2 Materials and methods

5.2.1. Construction of the pCAG-HAk DNA vaccine

5.2.1.1 Primer design

The oligonucleotide primers shown in Table 5.1 were used for amplification and sequencing of the AIV HA gene. Primers were designed to permit amplification of the full-length ORF of the HA of A/coot/WA/2727/79 (H6N2) containing a Kozak sequence. Thus, a Kozak sequence (GCCGCCACC) was inserted before the ATG start codon, an *Eco*RI site was added upstream of the Kozak sequence and a *Sma*I site was added to the

5’end of the downstream primer (HAd). These primers were synthesized by GeneWorks (GeneWorks Pty. Ltd.). Primers for the HA sequencing and pCAGGS primers were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd (Shanghai, China).

Table 5.1 Primers used for amplification and sequencing of AIV HA gene.

Primer	Sequence (5’to 3’)	Genome	Nt position*
HAu	ATGATTGCAATCATAATATTC	HA gene	16-36
HAd	TTATATACATATCCTGCATTG	HA gene	1696-1716
P1	CTGGGTAGCATATCCCATGT	HA gene	340-321
P2	AAATCCCCAATGTGACATCTTG	HA gene	251-272
P3	TGAGCAAAATACTCTGTATG	HA gene	620-639
P4	TAAGACTGGCAACTGGTCTGAG	HA gene	1000-1021
P5	ATGCCAATGTGAAGAACC	HA gene	1381-1398
pCAG-F	GAGATCTCGGAGACGATTG	pCAGGS	71-89
pCAG-R	CAGAAGTCAGATGCTCAAG	pCAGGS	97-78

* The nt position was based on the HA gene of A/coot/WA/2727/79 (H6N2) in the Appendix 2.1.

The pCAGGS expression vector was provided by Harbin Veterinary Research Institute, China and its structure is shown diagrammatically in Figure 5.1.

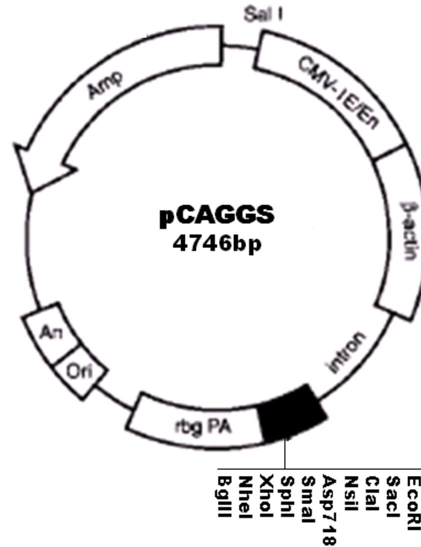

Figure 5.1 Schematic diagram of the pCAGGS vector.
Modified from Kodihalli *et al.*(1997). Ori refers to simian virus 40 origin of replication, CMV-IE/En refers to CMV/IE enhancer, β-actin refers to chicken β-actin promoter, rbg PA refers to rabbit β-globulin polyadenylation site, An refers to simian virus 40 antigen.

5.2.1.2 RNA extraction

Viral RNA was extracted using Trizol LS Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 250 µL of viral allantoic fluid was added to 750 µL Trizol LS Reagent in a 1.5 mL eppendorf tube. Following mixing and incubation at room temperature for 5 min, 200 µL chloroform was added and the mixture was vigorously shaken for 15 sec. After incubation for 10 min at room temperature, the mixture was centrifuged at 12,000 g for 10 min at 4°C. The supernatant (about 500 µL) was then transferred to a new tube followed by adding 500 µL isopropyl alcohol, and mixing and holding for 10 min at room temperature. After the centrifugation at 12,000 g for 10 min at 4°C, the supernatant was discarded and 1 mL 75% alcohol was added to the pellet followed by centrifugation at 12,000 g for 5 min at 4°C. Subsequent to discarding the

supernatant, the pellet was dried at room temperature and resuspended in 20 µL DEPC-treated dH_2O and then stored at -70°C until needed.

5.2.1.3 Reverse transcription

Reverse transcription was performed using Moloney murine leukaemia virus reverse transcriptase (Invitrogen) as described by the manufacturer. In brief, 20 µL RNA was mixed with 2.0 µL of 20 µM HAu. The mixture was heated at 70°C for 5 min to disrupt the secondary structure of the RNA and then quickly chilled on ice. After brief centrifugation, the following components were added up to a total volume of 40µL: 8.0µL of 5x first strand buffer, 4.0 µL of dNTP mix (2.5 mM each), 4.0 µL of 0.1M DTT, 1 µL of (40 U/µL) RNaseOUT (Invitrogen), and 1.0 µL(200 U/µL) M-MLV RT. Following mixing the reaction was held at 37°C for 3 hr and then stored at -70°C until needed.

5.2.1.4 Amplification of HA gene by proof-reading PCR

Proof-reading PCR was performed using the Phusion™ high-fidelity DNA polymerase protocol (Finnzymes OY, Espoo, Finland). Briefly, 50 µL PCR reactions contained 10 µL of 5x Phusion™ HF reaction buffer, 4 µL of 2.5 mM dNTP mix, 3 µL cDNA template, 1 µL of 20 µM HAu and 1 µL of 20 µM HAd, 0.5 µL (2U/µL) high-fidelity DNA polymerase, and 30.5 µL dH_2O. The thermal cycler program included initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation (10 sec at 98°C), annealing (1 min at 60°C) and elongation (45 sec at 72° C), and a final elongation step at 72°C for 5 min.

5.2.1.5 Restriction endonuclease digestion

Following agarose gel electrophoresis, the predicted PCR product of 1.7 kb was excised and recovered using a QIAquick Gel Extraction Kit (Qiagen) as previously described in section 2.2.13.

The recovered HA fragment and pCAGGS plasmid DNA was subjected to restriction endonuclease digestion with *Eco*RI (TaKaRa) and *Sma*I (Fermentas, Changsha, China) as described in section 4.2.1.2. The digestion product was purified using a PCR purification kit (Watson BioTechnologies Inc., Shanghai, China) and then quantified using Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech, Cambridge, UK).

5.2.1.6 Construction of pCAG-HAk expression vector

The *Eco*RI-*Sma*I digested HA fragment was ligated into the *Eco*RI-*Sma*I digested pCAGGS vector to generate pCAG-HAk as described in section 3.2.1.2.

Transformation was conducted as described in section 2.2.14.2 using DH5α competent *E. coli* cells (TaKaRa). The positive clones were screened by PCR and further confirmed by sequencing using the vector-specific primers.

5.2.1.7 Sequencing

A Beckman CEQ™2000XL DNA analysis system was used to perform sequencing. For this, 400 ng plasmid DNA and ddH_2O was added into a 0.2 mL thin-wall tube to a total volume of 7 μL, followed by denaturation at 96°C for 3 min. Subsequently 1 μL (5 pmol/μL) viral primer and 2 μL DTCS Quick Start Master Mix (Premix) (Beckman Coulter, CA, USA) were added and PCR was performed using the thermal cycler

program: 96°C for 20 sec, 50°C for 20 sec, 60°C for 4 min, 30 cycles followed by holding at 4°C.

The sequencing reaction was transferred to 2.5 μL stop solution (1 μL of 3 M NaAC (pH 5.2), 1 μL of 100 mM EDTA (pH 8.0), 0.5 μL of 20 mg/mL glycogen). Then 60 μL cold 95% (v/v) ethanol (-20°C) was added and the mixture was held at -20°C for 20 min, followed by centrifugation at 12,000 g at 4°C for 20 min. The pellet was subsequently washed twice with 200 μL cold 70% (v/v) ethanol (-20°C), followed by centrifugation at 12,000 g at 4°C for 10 min. The pellet was re-suspended in 30 μL formamide by holding at 4°C for 20 min. It was then transferred to the appropriate wells of the CEQ sample plate followed by overlaying with one drop of light mineral oil. The sample plate was loaded into the Beckman CEQ800 sequencer (Beckman Coulter).

After sequencing, Seqman from DNASTAR was used to edit and analyse the sequence and MegAlign was used to compare the result with previous HA sequencing results.

5.2.2 Large-scale preparation of plasmid DNA for use as a DNA vaccine

A large quantity of plasmid DNA was prepared according to the classical protocol (Sambrook and Russell 2001). Briefly, 1L bacterial culture was harvested by centrifugation at 5, 000 g for 15 min at 4°C. The pellet was dissolved in 100 mL STE (100 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)) buffer (4°C) followed by centrifugation at 5,000 g for 10-15 min at 4°C. The pellet was resuspended in 18 mL of solution I (4°C) followed by the addition of 2 mL of 10 mg/mL lysozyme (in 10 mM Tris (pH 8.0) buffer) (Bgi Life Tech Co., Ltd., Beijing, China). Then 40 mL of solution II was added to the suspension followed by gently mixing and incubation on ice for 10 min. Subsequently 30 mL of solution III (60 mL of 5 M KOAc, 11.5 mL glacial acetic acid

and 28.5 mL dH_2O) was added to the mixture and thoroughly mixed until a white precipitate was observed. After centrifugation at 8,000 g for 20 min at 4°C, the supernatant was filtered through 4-layers of sterile gauze. The filtered supernatant was mixed with 0.6 volume isopropanol followed by centrifugation at 8,000 g for 20 min at room temperature. The pellet was dissolved in 10 mL TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)) and then mixed with 10 mL of 5 M LiCl solution (4°C). The mixture was held on ice for 10 min before centrifugation at 10,000 g for 15 min at 4°C. The supernatant was mixed with an equal volume of isopropyl alcohol followed by holding at room temperature for 10 min. After centrifugation at 12,000 g for 10 min at 4°C, the pellet was rinsed with 70% ethanol. The pellet was then dissolved in 500 μL TE containing 20 μg/mL RNase A (Genebase Gene-Tech Co., Ltd., Shanghai, China) and held at 37°C for 30 min. The supernatant was mixed with an equal volume of phenol:cholorform:isoamyl alcohol (25:24:1) followed by centrifugation at 10,000 g for 5 min at room temperature. The aqueous phase was transferred to a polypropylene tube followed by the addition of an equal volume of cholorform. The supernatant was precipitated by addition of 1/10 volume 3M NaOAc (pH4.8) and 2.5 volumes 100% ethanol followed by incubation at -20°C for 30 min. After centrifugation at 15,000 g for 20 min at 4°C, the pellet was dissolved in 1 mL dH_2O followed by adding 0.5 mL PEG-$MgCl_2$ solution (40% (w/v) PEG8000, 30 mM $MgCl_2$), and the mixture was held at room temperature for 10 min. Following centrifugation at 12,000 g for 20 min at room temperature, the pellet was washed twice using 1 mL 70% ethanol followed by centrifugation at 12,000 g for 5 min. The pellet was dried at 37°C for 10 min, dissolved in 500 μL TE and stored at -20°C until required.

5.2.3 *In vitro* expression of pCAG-HAk

Transfection was conducted with Lipofectamine™ 2000 (Invitrogen Corp., CA, USA) following the manufacturer's instructions. Human embryonic kidney cell line 293T cells were grown in DMEM (Gibco BRL, CA, USA) supplemented with 10% FCS in a 6-well plate pre-coated with poly-lysine (Greiner-Bio One) until the cells were approximately 70-80% confluent. Then 5 µg plasmid DNA was added to 100 µL Opti-MEM (Gibco BRL) in a sterile tube and 10 µL (1mg/mL) Lipofectamine™ 2000 was added to 200µL Opti-MEM in another tube followed by gently mixing and holding at room temperature for 5 min. Subsequently, the Lipofectamine solution was added to the DNA solution followed by gently mixing and holding at room temperature for 30 min. During this period, the 6-well plate was washed twice with 2 mL Opti-MEM followed by the addition of 1.2 mL Opti-MEM into each well. The DNA-Lipofectamine mixture was then added to the cells, followed by incubation at 37°C for 6-10 hr. After the medium was removed, the cells were washed twice with Opti-MEM. The wells were filled with 1.5 mL Opti-MEM and the plate was incubated at 37°C for another 24 hr.

Thirty six to forty eight hours post-transfection the cells were fixed using 70% alcohol at room temperature for 30 min. Then the IFT was performed using chicken anti-H6 serum and rabbit anti-chicken FITC (Sigma-Aldrich, Beijing, China) as described in section 2.2.5. The monolayers were observed for specific green fluorescence under a Leica DMIRES2 microscope (Leica, Wetzlar, Germany).

The expression of pCAG-HAk in Cos-7 cells and subsequent detection of HA antigen bands by WB was previously described in sections 3.2.4 and 3.2.5.

5.2.4 Animals and immunizations

Chicken vaccination and challenge studies were conducted as described in section 4.2.3 using SPF chickens at the Harbin Veterinary Research Institute, China.

For the vaccinations, plasmid DNA was diluted in PBS to deliver the doses indicated in Table 5.2 and 5.3 as a 200 µL volume administered in the leg muscles by either IM injection or EP, as described in section 4.2.3. Three groups of 8 chickens received either 10, 100 or 300 µg pCAG-HAk by IM injection (Groups 1-3); one group of 8 chickens received 100 µg pCAGGS by IM injection (Group 4) and one group of 8 chickens received 100 µg pCAG-HAk by electroporation (Group 5). Three weeks later, these five groups of birds were given booster vaccinations using the same dose and delivery route. Another group of 15 chickens (Group 6) received only a single vaccination of 100 µg pCAG-HAk by IM injection.

5.2.5 Virus challenge

In preparation for challenge studies with pCAG-HAk-vaccinated chickens, a pilot study was conducted to determine clinical signs and virus shedding in control chickens using the H6N2 LPAI challenge virus available in Harbin, since A/coot/WA/2727/79 (H6N2) virus was unavailable. Six 8-week-old naïve SPF chickens were challenged with a $10^{6.17}$ EID_{50}/0.1 mL dose (HA titre 2^6) of A/duck/Anhui/269/2002 (H6N2) virus in a 0.2 mL volume via nasal instillation.

OS and CS were collected from all chickens at 1, 3, 5 and 7 days post-challenge for virus isolation in chicken embryonated eggs. The chickens were observed daily for clinical signs and death over a 2-week period.

Sera were collected at day 10 post-vaccination for detection of HI antibody as described in section 2.2.3.3.

Statistical analysis of data was conducted as per section 2.2.18.

5.3 Results

5.3.1 Confirmation of the pCAG-HAk construct

Because no antibodies or marginal antibody response in vaccinated chickens in previous chapters may have been due to mutation of the HA gene, the new HA DNA construct was developed directly from the H6N2 virus. The HA fragment was subsequently amplified by proof-reading PCR, using an annealing temperature of 60°C, to yield a product of 1.7 kb. After cloning of the HA gene into the pCAGGS vector, the inserted gene was sequenced using the primers (P1-P5) in Table 5.1. The HA sequence was consistent with that achieved in Chapter 2.

5.3.2 *In vitro* expression of the pCAG-HA construct

Forty eight hours after transfection of 293T cells with pCAG-HAk and pCAGGS, the cell monolayers were stained by IFT. Specific fluorescence was mainly distributed in the cell membranes of cells transfected with pCAG-HAk as shown in Figure 5.2.

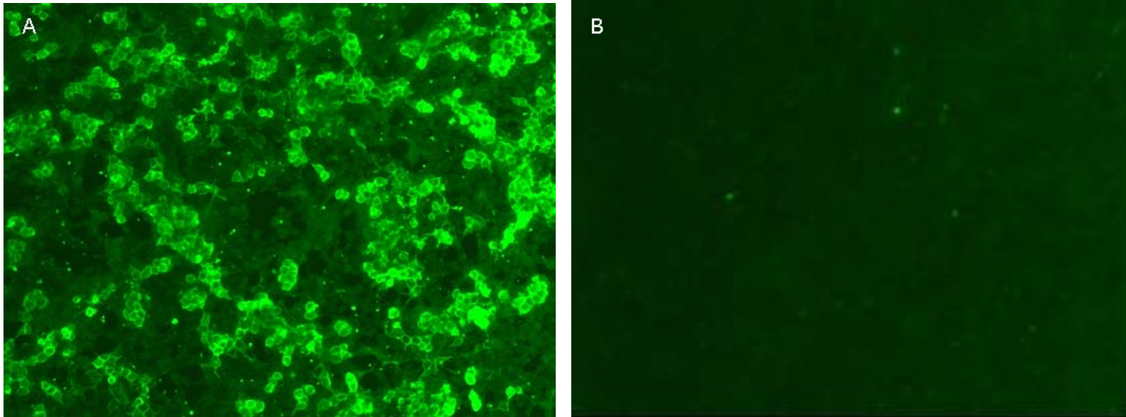

Figure 5.2 Immunofluorescence in 293T cells after transfection with the pCAG- HAk (A) and absence of specific staining with pCAGGS (B).(x100).

The HA protein expression in pCAG-HAk transfected Cos-7 cells was detected by WB, displaying a strong band of approximately 78 kDa (Figure 5.3).

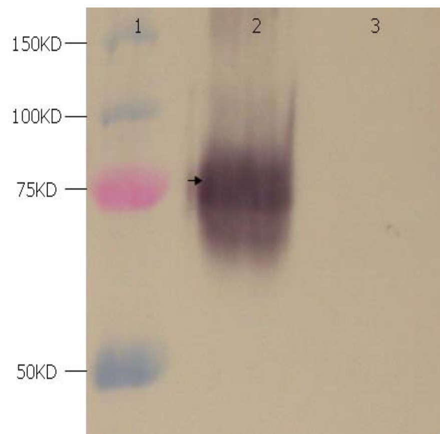

Figure 5.3 HA expression in Cos-7 cells detected by WB.
Lane 1, protein markers (Bio-Rad); lane 2, pCAG-HAk transfected cells; lane 3, pCAGGS transfected cells.

5.3.3 Antibody response induced by two vaccinations of pCAG-HAk construct

As shown in Table 5.2 (Groups 1 to 3), the earliest time of sero-conversion appeared two weeks after vaccination. The H6 HI test sero-conversion rates three weeks after initial vaccination were 37.5%, 12.5% and 0 in the 10, 100 and 300 μg pCAG-HAk vaccinated groups respectively. After the booster vaccination the sero-conversion rate increased to 87.5%, 75% and 75% respectively, indicating that a second vaccination increased the sero-conversion rate in all three groups.

The profile of HI antibodies induced by different doses of pCAG-HAk (Groups 1 to 3) during the 8-week study period was similar (Table 5.3 and Figure 5.4). After one dose, the vaccinated birds in the 10 μg and 100 μg pCAG-HAk groups demonstrated HI antibody, but those in the 300 μg group did not. However, the H6 HI titre rose significantly in the three different dose groups following booster vaccination and reached a plateau 2-3 weeks post-booster. No H6 HI titre was observed in the pCAGGS vaccinated group. The GMT of HI antibody was significantly different (Mann-Whitney Test, $p<0.05$) between the combined pCAG-HAk groups and the pCAGGS group at weeks 4, 5, 6, 7 and 8 following initial vaccination, but there was no significant difference at week 3 after vaccination. There was no significant difference in GMT between the three doses of pCAG-HAk vaccine (ANOVA, $P = 0.963$).

Table 5.2 Sero-conversion rate by H6 HI test in chickens immunized with pCAG-HAk or pCAGGS.

Group	Vaccine	Dose (μg)	Route	Sero-conversion rate (weeks post vaccination)											
				1	2	3*	4	5	6	7	8	9	10	11	12
1	pCAG-HAk	10	IM	0/8**	1/8	3/8	4/8	6/8	6/8	7/8	7/8				
2	pCAG-HAk	100	IM	0/8	1/8	1/8	2/8	6/8	5/8	5/8	6/8				
3	pCAG-HAk	300	IM	0/8	0/8	0/8	5/8	6/8	6/8	6/8	5/8				
4	pCAGGS	100	IM	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8				
5	pCAG-HAk	100	EP	1/8	5/8	7/8	8/8	8/8	8/8	8/8	8/8				
6	pCAG-HAk	100	IM	0/15	6/15	5/15	7/15	10/15	8/15	8/15	8/15	8/15	8/15	8/15	8/15

*Time of booster vaccination for Groups 1 to 5; Group 6 was only vaccinated once.
**N/N represents HI antibody positive chickens/all vaccinated chickens.

Table 5.3 HI titres in chickens immunized with pCAG-HAk or pCAGGS via IM or EP over an 8- or 12- week period after vaccination.

Vaccine	Dose (μg)	Route	HI titre (weeks post vaccination)											
			1	2	3*	4	5	6	7	8	9	10	11	12
pCAG-HAk	10	IM	0**	0.4±1.1	0.9±1.4	1.6±2.1	2.4±2.1	2.8±2.1	3.1±2.0	3.2±2.1				
pCAG-HAk	100	IM	0	0.4±1.1	0.4±1.1	1.0±2.1	2.6±2.6	2.9±2.9	2.9±2.7	2.9±2.3				
pCAG-HAk	300	IM	0	0	0	1.5±1.7	2.6±2.2	2.6±2.0	2.3±1.7	1.9±1.8				
pCAGGS	100	IM	0	0	0	0	0	0	0	0				
pCAG-HAk	100	EP	0.1±0.4	3.3±3.4	3.8±2.8	6.7±3.0	7.1±3.0	6.6±2.3	5.8±2.4	5.6±2.1				
pCAG-HAk	100	IM	0	1.0±1.4	0.9±1.4	2.0±2.5	2.4±2.3	2.1±2.2	1.8±1.9	1.7±1.8	1.5±1.6	1.3±1.3	1.1±1.2	1.1±1.2

* Chickens in Groups 1-5 were given a booster 3 weeks after initial immunization; Group 6 was only vaccinated once.
** H6 HI antibody represents GMT (log_2) ± SD.

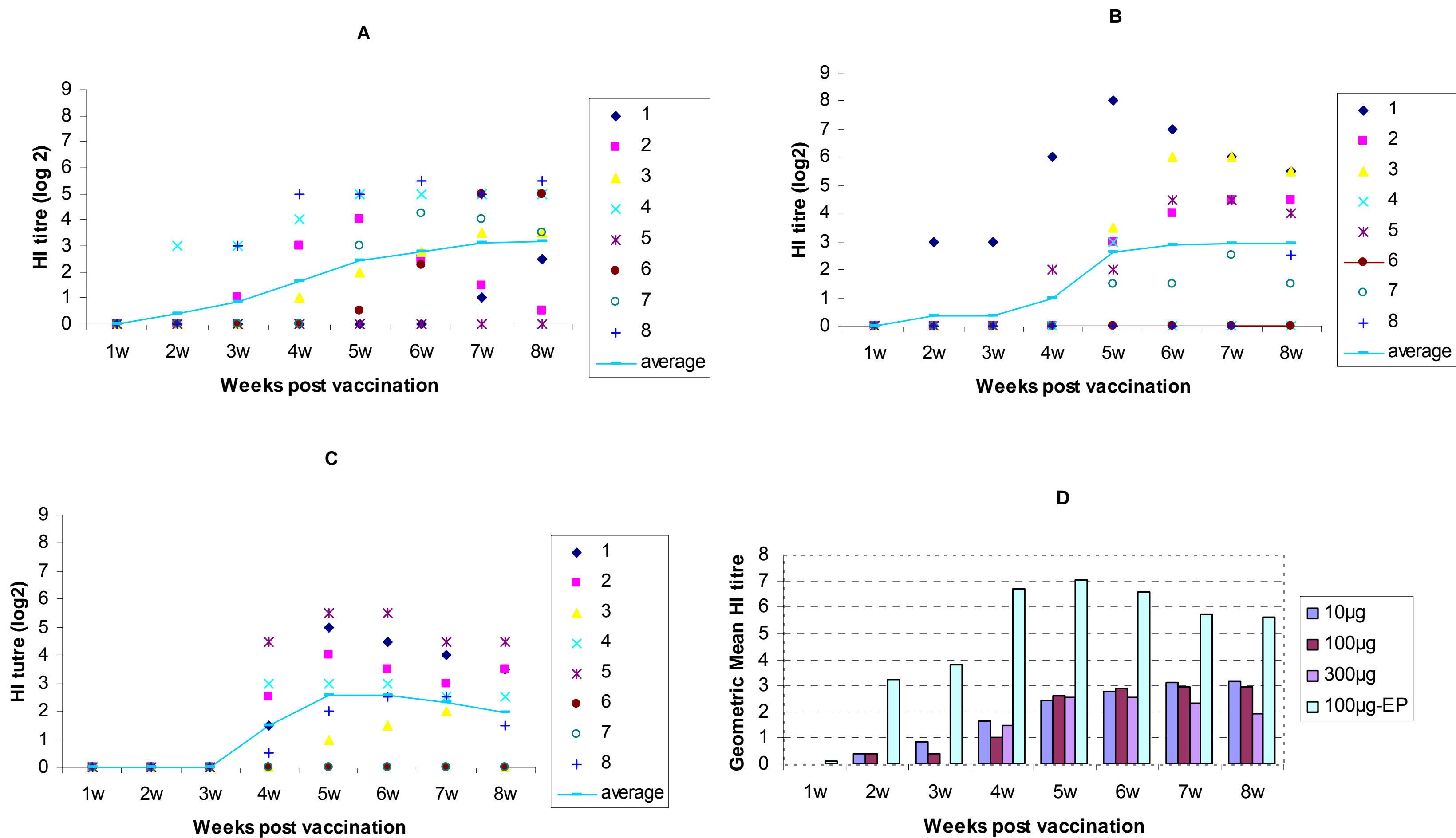

Figure 5.4 Temporal changes of H6 HI titers over an 8 week period post-vaccination in chickens immunized with 10 µg (A), 100 µg (B) and 300 µg (C) pCAG-HAk. The curve represents the mean antibody titre of the relevant group. Numbers refer to individual birds.(D) Average H6 HI antibody titres in the 10, 100, 300 µg pCAG-HAk groups via IM vaccination and the 100 µg pCAG-HAk group vaccinated with EP.

5.3.4 Antibody response induced by a single vaccination of the pCAG-HAk construct

Of 15 birds vaccinated with a single dose of 100 µg pCAG-HAk construct, 40 % (6/15) sero-converted at week 2 post-vaccination and 66.7% (10/15) at week 5 post-vaccination (Group 6 in Table 5.2). The peak HI GMT occurred at week 5 post-vaccination and the HI titre slowly declined during the 3-month observation period (Figure 5.5). There were no significant differences (ANOVA, P>0.05) in the GMT of H6 HI antibody at weeks 2 and 3 post-vaccination between Group 6 and the pCAGGS vector group (Group 4). Neither was there a significant difference in the HI antibody GMT (ANOVA, P=0.43) or in the sero-conversion rate (Fisher's exact test, P=0.37) at week 3 following initial vaccination between Group 6 and Group 2 (Table 5.2 and Table 5.3).

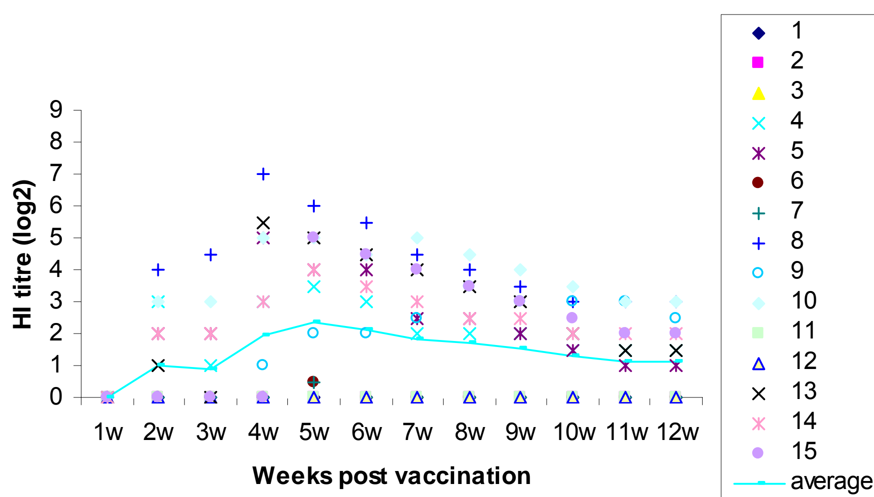

Figure 5.5 Temporal changes in H6 HI titer over a 12-week post-vaccination period in chickens immunized with a single dose of 100 µg pCAG-HAk.
The curve represents the mean antibody titre of the group. Each icon number refers to one bird.

5.3.5 Antibody response induced by two vaccinations of pCAG-HAk construct via EP

Birds administered pCAG-HAk construct via EP showed 87.5% sero-conversion after the first vaccination and 100% sero-conversion after the second vaccination (Group 5 in Table 5.2). The highest HI antibody GMT ($2^{7.1}$) occurred at week 5 post-vaccination then the antibody gradually declined. The highest HI titre for an individual bird was 2^{11} (Figure 5.6).

There were significant differences (ANOVA/Mann-Whitney Test, P<0.05) in the HI antibody GMT from week 2 to 8 between the IM and EP groups receiving 100 µg pCAG-HAk (Figure 5.5d).

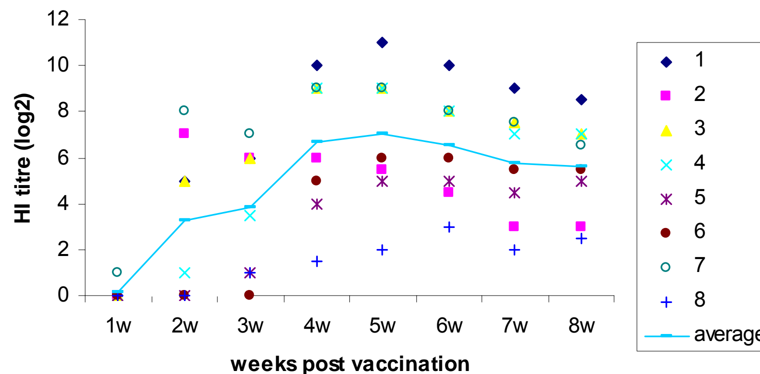

Figure 5.6 Temporal changes in H6 HI titer over an 8-week period post-vaccination in chickens immunized with 100 µg pCAG-HAk via EP.
The curve represents the mean antibody titre of the group. Each icon number refers to one bird.

5.3.6 Virus challenge

Six naive SPF chickens challenged with A/duck/Anhui/269/2002 (H6N2) virus did not exhibit any clinical signs during the two-week period post-challenge. Only three birds shed the virus intermittently in OS in the first four days, whereas, no virus shedding was detected in CS during 7 consecutive days of sampling. The GMT of H6 HI antibody at

day 10 post-challenge was $2^{4.67}$ (range from $2^{2.5}$ to 2^{6}) using homologous virus (A/duck/Anhui/269/2002) as antigen compared with $2^{1.25}$ (range from 0 to $2^{2.5}$) using heterologous virus (A/coot/WA/2727/79) as antigen.

5.4 Discussion

This study successfully demonstrated that a pCAG-HAk DNA vaccine was able to elicit measurable H6 HI antibody responses. Either one or two vaccinations of naked plasmid DNA induced sero-conversion in a substantial proportion of chickens vaccinated. In particular, 100% of chickens vaccinated with the pCAG-HAk DNA vaccine via EP generated high titres of H6 antibody, which is comparable to titres previously achieved with an inactivated whole virus H6N2 vaccine (Table 3.3 in Chapter 3). Nevertheless, the higher dose (300 µg) of pCAG-HAk DNA administered by IM injection did not produce any higher antibody responses than the lower doses used (either 10 µg or 100 µg).

The temporal antibody response studies following pCAG-HAk vaccination with EP showed that the earliest detectable antibody was at one week post-vaccination, compared with two weeks after IM administration. However, in some IM vaccinated chickens the immune response was not detectable until five weeks post-vaccination. This long lag phase is probably related to poor DNA uptake into muscle fibers via IM delivery, the mechanism of which is poorly understood. It has been hypothesized that IM injection of naked DNA vaccines only causes the transfection of a limited number of muscle fibres however, due to the low rate of muscle fibre turnover, this can result in the expression of the plasmid DNA for an extended period of time (Pertmer *et al.* 1996; Wolff *et al.* 1990). In addition, myocytes are terminally differentiated, long-lived cells that cannot re-enter mitosis, and thus do not dilute out the internalized plasmids resulting in long-term

stability and transcriptional activity of plasmid DNA (Fattori *et al.* 2002; van Deutekom *et al.* 1998).

The antibody titre peaked at approximately two weeks following two DNA vaccinations via either IM or EP, persisted for several weeks and gradually declined. With IM administration, two immunizations appeared to be better than one in terms of the strength and persistence of the antibody response. The effect of a single immunization via EP was not evaluated in this study due to an inadequate number of SPF isolators and the limited experimental period available.

Dose–response studies demonstrated that IM injection of pCAG-HAk DNA at doses of 10 µg, 100 µg or 300 µg could induce HI antibodies. Although the sero-conversion rate following two vaccinations was similar in the three different dose groups, both 10 µg and 100 µg groups appeared to generate higher H6 HI geometric antibody titres than the 300 µg group, although this was not significantly different. This result suggested that lower dose DNA vaccination appeared to generate a better antibody response than vaccination with a higher dose and this is similar to observations made in Chapters 3 and 4. In addition, the observation that vaccination with the 10 µg DNA plasmid dose via the IM route appeared to induce similar H6 HI antibody titres to the 100 µg DNA plasmid dose has implications for the cost-effectiveness of such DNA vaccines.

Although GG delivery system has been shown to reduce the dosage required to protect chickens against H5 AI to 1–10 µg plasmid DNA (Kodihalli *et al.* 1997), this method is impractical for field use. The present study indicated that IM injection at a dose of 10 µg induced a similar antibody response to 100 µg DNA vaccination. This was in agreement with a research report (Ulmer *et al.* 1994), in which IM injection of low doses of HA or

NP-expressing DNA at doses of 100 ng to1 µg was successful in generating antibodies to HA and CTL respectively, and in protecting mice against influenza virus challenge.

In the group receiving a single dose of the 100 µg pCAG-HAk DNA vaccine, the antibody response at week 3 post-vaccination appeared stronger in terms of the sero-conversion rate than in the group with two injections of the same dose three weeks apart. However, the differences between the groups at week 3 post-vaccination were not significantly different and probably resulted from biological variation in individuals within the experimental groups. In addition, there was considerable individual variation in H6 HI antibody response among chickens receiving the pCAG-HAk vaccine intramuscularly. The highest H6 HI titre reached was 2^8, whereas, some birds had no measurable antibody response. Similarly, in the EP group, the highest titre achieved was 2^{11} and the lowest, after two doses, was 2^2. This variation has also been reported in other DNA vaccine studies in chickens (Suarez and Schultz-Cherry 2000a) and mice (Ulmer *et al.* 1994). The reason for the wide variation in individual HI antibody titres in vaccinated birds is not clear, but may be attributable to the poor efficiency of the delivery method (Wang *et al.* 2008b). This inter-individual variation is another drawback of DNA vaccines and supports the need to fully elucidate the mechanism of the DNA-induced immune response.

For this study the intention was to conduct a heterologous H6N2 challenge with pCAG-HAk vaccinated chickens using A/duck/Anhui/269/2002 virus that was available in Harbin and then to conduct a homologous challenge in chickens vaccinated with pCAG-HAk at Murdoch University. However, the pilot trial of the heterologous challenge in naive control birds with the H6N2 virus available in Harbin produced no clinical signs

and very limited virus shedding. Thus, group sizes would have to have been large to detect any differences in virus shedding between vaccinates and controls with this virus and as time constraints were in place the heterologous challenge study was not conducted at Harbin. This challenge would have been used to optimize the pCAG-HAk vaccine dose for the homologous challenge studies at Murdoch University.

Although the 10 µg pCAG-HAk plasmid dose administered intramuscularly provided similar antibody responses to the 100 µg DNA dose in this study, it was decided to use 100 µg pCAG-HAk plasmid dose for the DNA vaccine studies performed at Murdoch, reported in Chapters 7 and 8, because of the variability of plasmid uptake by muscle cells and the DNA plasmid dosage used by other DNA vaccine researchers (Chen *et al.* 2001; Cherbonnel *et al.* 2003).

It has been established previously that chickens given inactivated vaccines that reach a post-vaccination geometric mean HI titre of greater than $2^{6.5}$ had good protection after challenge, based on disease signs and tracheal/cloacal virus isolation (Wood *et al.* 1985). However, in vaccine challenge studies using field vaccinated birds with a H5 HI titre of 2^5 or higher there was complete protection against a high challenge dose of Asian H5N1 HPAI (Ellis *et al.* 2004). A close relationship between HI titers generated by plasmid DNA (greater than 1:40) and protection was observed previously in mice, ferrets and non-human primates immunized with a plasmid encoding consensus HA gene of H5 AIV (Laddy *et al.* 2008). Although LPAI virus challenge was not able to be conducted in the Harbin studies, if protection/reduction in virus shedding is equated with a HI titre of 2^5 or higher, at least six out of eight chickens in the 100 µg EP group and two out of eight

birds in the 100 µg IM group, with HI titres greater than 1:64, had achieved this level of protection.

In vivo electroporation has been well documented to improve the efficacy of DNA vaccination in eliciting both humoral and cellular immune responses in several models including mice (Chen *et al.* 2005; Dobano *et al.* 2007; Dupuis *et al.* 2000; Hooper *et al.* 2007; Widera *et al.* 2000), goats and cattle (Tollefsen *et al.* 2003), sheep (Babiuk *et al.* 2002; Babiuk *et al.* 2007; Tsang *et al.* 2007), rabbits (Wang *et al.* 2008b) and non-human primates (Luckay *et al.* 2007). In this study, the mean H6 HI titre was ≥ 8-fold higher in the EP vaccinated group than in the IM vaccinated group from 2 to 8 weeks post-vaccination. This indicated that vaccination with EP was significantly more effective than routine IM inoculation with the pCAG-HAk vaccine in chickens.

The promoter driving gene expression was reported previously to be one of the factors affecting the efficacy of DNA vaccines (Xiang *et al.* 1995). Depending on their origin, promoters differ in tissue specificity and efficiency in initiating mRNA synthesis (Kodihalli *et al.* 1997; Xiang *et al.* 1995). One study demonstrated that CMV and chicken β-actin promoters were equally suitable for use with a H5 HA-expressing DNA vaccine in chickens (Kodihalli *et al.* 1997), on the basis of protection against lethal challenge with H5N1 HPAI virus. However, in contrast to the previous CMV promoter-driven HA-expressing constructs described in Chapters 3 and 4, the pCAG-HAk plasmid containing the chicken β-actin promoter was more effective at producing H6 HI antibody responses in chickens.

The Kozak sequence plays a major role in the initiation of a translation process in mammalian cells (De Angioletti *et al.* 2004). As prokaryotic genes and some eukaryotic

genes do not have Kozak sequences, it has been postulated that the expression level of these genes might be augmented by the insertion of a Kozak sequence. Insertion of a Kozak sequence upstream of the gene in a pUC vector was shown to significantly increase *in vitro* expression of the human serum albumin gene in horse cells (Olafsdóttir *et al*., 2008). Thus, this approach would appear to be a useful and practical tool in the development of improved DNA vaccines.

In this study, a pCAG-HAk DNA vaccine was constructed that incorporated a Kozak sequence upstream of the ATG initiation codon of the HA gene. Insertion of the Kozak sequence and use of the high-efficiency pCAGGS vector synergistically contributed to a higher level of H6 HA expression than other plasmid vectors evaluated in earlier chapters, resulting in a detectable antibody response. Analyzing the parameters which impact on DNA-induced antibody responses is important to successfully develop DNA vaccines. Further investigation of these factors in this DNA construct was carried out in the following chapters to identify which plays the more critical role in eliciting antibody responses.

Chapter 6

Evaluation of codon usage on the immunogenicity of a HA DNA vaccine against H6N2 avian influenza

6.1 Introduction

DNA immunization has emerged as a promising new approach to vaccination. However, successful DNA vaccination requires high expression of genes derived from microorganisms in animals and humans. One of the barriers for expression of genes derived from microorganisms in mammalian cells may be attributable to the inter-specific difference of codon usage (Nagata *et al.* 1999). It is known that mammalian codon usage patterns are different from those of numerous microorganisms and yeasts (Ikemura 1985). Thus, differences in codon usage between a heterologous gene and the host organism may have an impact on expression of the gene, which may eventually affect the immunogenicity of an antigen-encoding DNA vaccine.

A codon is a trinucleotide sequence encoding an amino acid residue. Most amino acids are encoded by more than one codon as the genetic code is degenerate. However, the choice of codons is not random and the frequency of synonymous codon usage is biased depending on the species (Makoff *et al.* 1989; Nagata *et al.* 1999). Thus, codon usage optimization of a gene, which substitutes a mammalian-biased codon for the codon preference of a microorganism, represents a novel strategy for engineering a DNA vaccine construct or enhancing the efficacy of DNA vaccination. Nagata *et al.*(1999) examined how codon optimization affected the translational efficiency in mammalian cells using plasmid DNA encoding a CTL epitope derived from *Listeria monocytogenes* and *Plasmodium yoelii*. It was reported that codon optimization of the green fluorescent protein gene (Zolotukhin *et al.* 1996) or human erythropoietin gene with human high-

frequency codons gave a considerably higher level of expression in mammalian cells. It was also reported that codon optimization was effective in enhancing the immunogenicity of DNA vaccines against influenza virus (Wang *et al.* 2006), H5 AIV (Jiang *et al.* 2007), HIV (Andre *et al.* 1998; Deml *et al.* 2001; Kumar *et al.* 2006), papillomavirus (Cheung *et al.* 2004); *Clostridium tetani* (Stratford *et al.* 2000), *Mycobacterium tuberculosis* (Ko *et al.* 2005) and *Plasmodium falciparum* (Narum *et al.* 2001). Codon optimization was also used to develop DNA vaccines against emerging diseases such as Nipah virus (Wang *et al.* 2008c), Chikungunya virus (Muthumani *et al.* 2008) and severe acute respiratory syndrome (SARS) virus (Yang *et al.* 2004).

The purpose of the study reported in this chapter was to construct a DNA plasmid encoding a codon optimized HA gene and to determine if it could maximise immunogenicity of a H6 HA-expressing DNA vaccine in chickens.

6.2 Materials and Methods

6.2.1 Preparation of a codon-optimized HA-expressing DNA vaccine construct

The chicken biased codon information (the codon preference of *Gallus gallus*) was obtained from a codon usage database (http://www.kazusa.or.jp/codon/). The sequence of the full-length HA gene from A/coot/WA/2727/79 (H6N2) was shifted to the chicken-biased codons using Editseq of DNASTAR. Despite DNA-level sequence changes, the final codon-optimized HA DNA sequence still produced the same HA amino acid sequences as those in the wild type AI virus. The Kozak sequence GCCGCCACC was added immediately before the start codon. For subcloning purposes the restriction endonuclease sites of *Eco*RI and *Sma*I were added immediately upstream of the Kozak sequence and downstream of the stop codon, respectively. This codon-optimized HA

gene was chemically synthesized and cloned into pUC57 (Figure 6.1) by GenScript Corporation (Beijing, P.R. China), with the addition of an *Eco*RV site.

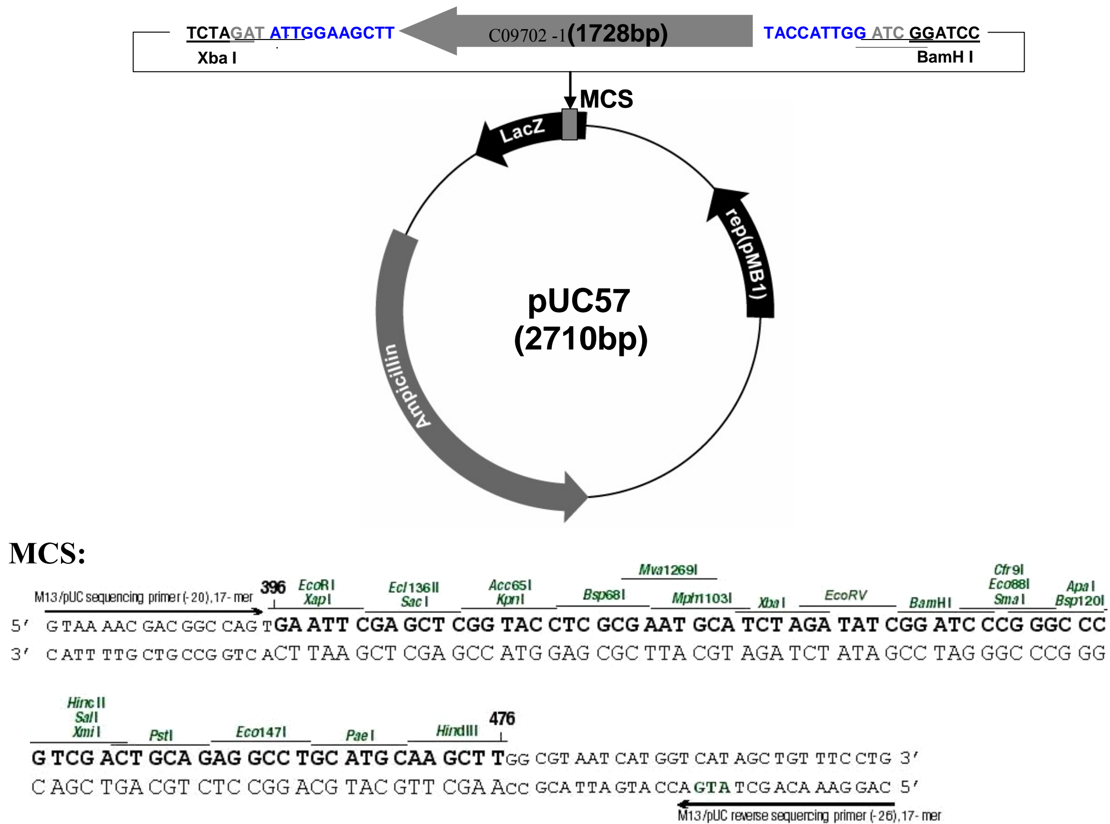

Figure 6.1 Plasmid construct map from GenScript Corporation (Beijing, China).
The C09702-1 (optiHA gene) was cloned in pUC57 by *Eco*RV. Blue sequences refer to protective bases added by GenScript.

The synthesized codon-optimized HA gene insert was subcloned into the expression vector pCAGGS to generate pCAG-optiHAk at the *Eco*RI and *Sma*I sites as described in Chapter 5. This ligation product was transformed into *E. coli* (H5α strain). The positive clone was selected based on the results of PCR and restriction enzyme digestion of the

DNA construct. Plasmid DNA was prepared for *in vitro* transfection using a QIAprep®Spin Miniprep kit (Qiagen) as described in section 2.2.15 and for *in vivo* animal immunization using the classical alkaline lysis and subsequent PEG purification as described in section 5.2.2.

6.2.2 Confirmation of *in vitro*-expressed HA antigens

The pCAG-optiHAk construct was transiently transfected into 293T cells or BSR (a clone of BHK-21) using Lipofectamine™ 2000, as described in section 5.2.3. The expression of HA antigens was verified by WB and IFT as described in sections 3.2.5.3 and 2.2.5 respectively. The substrate for WB was 3, 3'-diaminobenzidine (DAB) (Boster Biotechnology Co. Ltd, Wuhan, China).

6.2.3 DNA immunization of chickens

Groups of 6-week-old SPF chickens housed in HEPA-filtered isolators, with 8 birds per group, were intramuscularly injected with either 10 µg or 100 µg pCAG-optiHA plasmid as per section 5.2.4. This experiment commenced three weeks after the study in Chapter 5 using SPF chickens from the same batch as Chapter 5. Sera were collected on a weekly basis post-vaccination for HI assays as per section 2.2.3. Statistical analysis of data was conducted as per section 2.2.17.

6.3 Results

6.3.1 Effect of codon optimization on HA composition

The chemically synthesized chicken codon-biased HA gene from A/ coot/WA/2727/79 (H6N2) had the same HA amino acid sequences as the original virus, but the nucleotide homology between the wild-type HA gene and the codon-optimized HA gene was 74.8%.

The comparison at nucleotide sequence level between them is shown in Appendix 6.1 using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw/). A summary of the codon preference changes before and after codon optimization using EditSeq of DNASTAR is presented in Table 6.1. Codon optimization resulted in an increase in the G+C content from 41.3% in HA wild-type to 59.5% after optimization.

Table 6.1 Comparison of the most frequently prevalent codons in the chicken and H6N2 virus.

Amino acid	Codon		Amino acid	Codon	
	Virus	Chicken		Virus	Chicken
Ala	GCA	GCC	Met	AUG	AUG
Arg	AGA	AGA	Leu	UUG	CUG
Asn	AAU	AAC	Lys	AAA	AAG
Asp	GAU	GAU	Phe	UUC/UUU	UUC
Cys	UGU	UGC	Pro	CCA	CCC
Gln	CAA	CAG	Ser	UCA	AGC
Glu	GAA	GAG	Thr	ACA	ACC
Gly	GGA	GGC	Trp	UGG	UGG
His	CAU	CAC	Tyr	UAU	UAC
Ile	AUA	AUC	Val	GUG	GUG

6.3.2 Identification of insert gene by restriction endonuclease digestion and PCR

The synthesized gene was sequenced by the company. For confirmatory identity, the synthesized codon optimized HA gene in a pUC57 vector was digested by *Eco*RI and *Sma*I, resulting in a 1.7 kb HA fragment and a 2.71 kb pUC57 fragment. The pCAG-optiHAk construct was identified by PCR using virus primers and a vector/virus primer pair, and restriction endonuclease digestion (Figure 6.2).

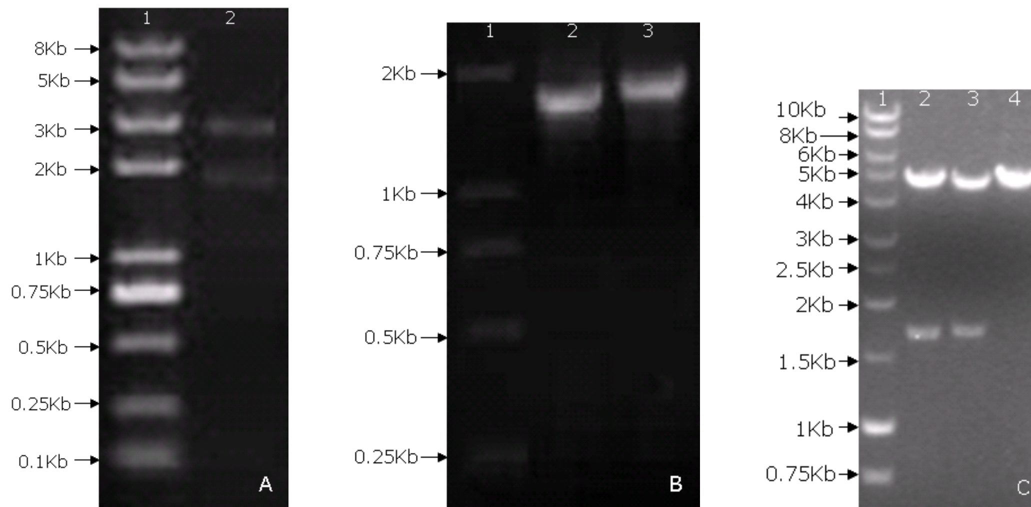

Figure 6.2 A Identification of synthesized optiHA gene by restriction endonuclease digestion. Lane 1, DL2000 plus DNA marker (Beijing Tuoyingfang Biotech Co.,Ltd , Beijing, China); lane 2, synthesized optiHA (*Eco*RI/*Sma*I). **B Identification of optiHA gene by PCR.** Lane 1, DL2000 marker; lane 2, optiHA fragment (1.7 kb) amplified by HAu/HAd primers; lane 3, optiHA fragment (1.8 kb) amplified by HAu/pCAG-R primers. **C Restriction enzyme analysis of optiHA gene.** Lane1, 1 kb marker; lane 2, pCAG-optiHAk (*Eco*RI/*Sma*I); lane 3, pCAG-HAk (*Eco*RI/*Sma*I); lane 4, pCAGGS.

6.3.2 Transient expression of pCAG-optiHAk construct

The pCAG-optiHAk construct and pCAGGS vectors transfected into 293T cells were fixed by alcohol at 48 hours and the encoded antigen expression was detected by IFT. Specific fluorescence was clearly observed, mainly distributed in the cell membrane in pCAG-optiHAk transfected 239T cells, but not in pCAGGS transfected cells (Figure 6.3). The outcomes of transient transfection of the synthetic and native HA genes in BSR cells were also compared by WB. As illustrated in Figure 6.4, both the pCAG-optiHAk and the pCAG-HAk (wildtype) transfected BSR cells showed approximately 84 kD bands of the expected size, but the pCAG-optiHAk construct did not produce better antigen expression *in vitro* than the pCAG-HAk (wildtype).

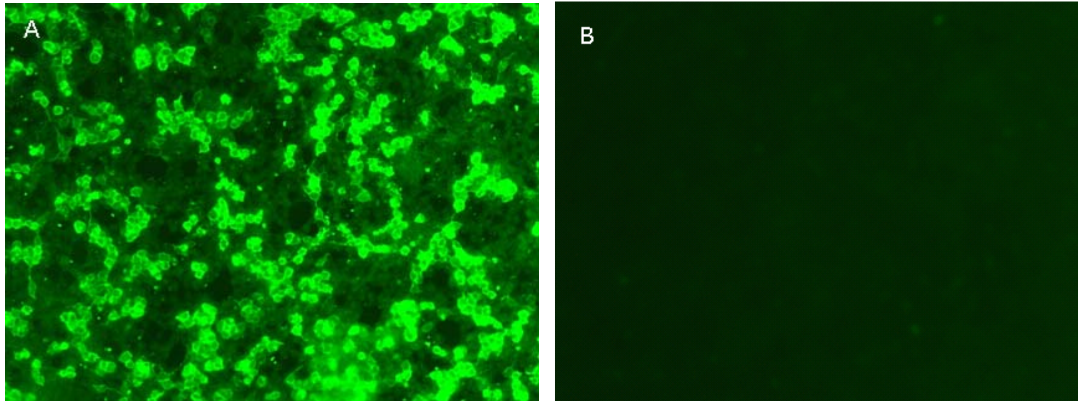

Figure 6.3 293T cells transfected with pCAG-optiHA (A) and with pCAGGS (B) (x100).

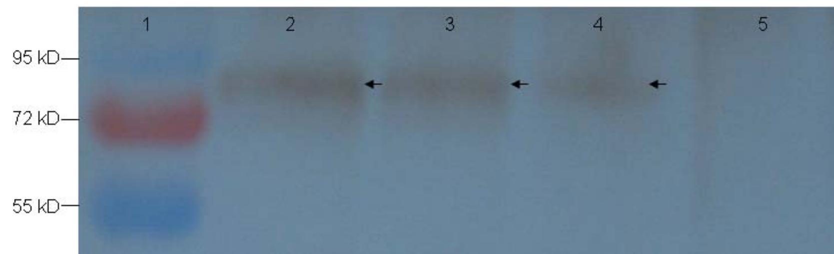

Figure 6.4 Western blotting analysis of HA protein expressed in BSR cells.
Lane 1, marker (PageRulerTMPrestained Protein Ladder, Fermentas, MD, USA); lane 2, pCAG-optiHAk; lane 3, pCAG-HAk; lane 4, pCI-HAk; lane 5, pCAGGS.

6.3.3 Antibody response

The kinetics of the antibody response induced by pCAG-optiHAk is shown in Figure 6.5 and Table 6.3. Following booster vaccination, the seroconversion rate and HI titres increased in both groups. The higher dose groups generated better immune responses than the lower dose groups in terms of the seroconversion rate, although there were no significant differences (P>0.05) in the GMT of HI antibody between different doses of pCAG-optiHAk. This suggested that a 100 μg dose of pCAG-optiHAk was a more optimal dosage for induction of HI antibody responses.

Table 6.2 Sero-conversion rates in chickens immunized with pCAG-optiHAk, pCAG-HAk and pCAGGS*.

Group	Vaccine	Dose (µg)	Route	Sero-conversion rate (weeks post vaccination)								
				1	2	3**	4	5	6	7	8	9
1	pCAG-optiHAk	10	IM	0/8*** ^{a}	0/8^{a}	1/8 ^{a}	1/8 ^{a}	2/8 ^{a}	3/8 ^{a}	3/8 ^{a}	2/8 ^{a}	2/8 ^{a}
2	pCAG-optiHAk	100	IM	0/8 ^{a}	2/8 ^{a}	3/8 ^{a}	3/8 ^{a}	4/8 ^{a}	4/8 ^{a}	7/8 ^{a}	7/8 ^{b}	7/8 ^{b}
3	pCAG-HA	10	IM	0/8 ^{a}	1/8 ^{a}	3/8 ^{a}	4/8 ^{a}	6/8 ^{a}	6/8 ^{a}	7/8 ^{a}	7/8 ^{b}	
4	pCAG-HA	100	IM	0/8 ^{a}	1/8 ^{a}	1/8 ^{a}	2/8 ^{a}	6/8 ^{a}	5/8 ^{a}	5/8 ^{a}	6/8 ^{ab}	
5	pCAGGS	100	IM	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

* Data from the Chapter 5 for comparison purposes. ** Time of booster vaccination. *** N/N represents HI antibody positive chickens in all vaccinated chickens. Within the column the different lowercase superscript letter indicates statistical differences ($P < 0.05$) using Fisher's exact test, group 5 excluded in statistical analysis.

Table 6.3 HI antibody in chickens immunized with pCAG-HAk, pCAG-optiHAk and pCAGGS.

Group	Vaccine	Dose (µg)	Route	HI titre (weeks post vaccination)								
				1	2	3*	4	5	6	7	8	9
1	pCAG-optiHAk	10	IM	0	0	0.1±0.2**	0.4±1.1	1.3±2.5	1.4±2.5	1.1±2.0	1.2±2.2	1.2±2.2
2	pCAG-optiHAk	100	IM	0	1.1±1.5	0.8±1.3	1.4±1.9	2.5±2.7	2.6±2.8	3.2±2.3	2.9±2.2	3.0±2.1
3***	pCAG-HAk	10	IM	0	0.4±1.1	0.9±1.4	1.6±2.1	2.4±2.1	2.8±2.1	3.1±2.0	3.2±2.1	
4***	pCAG-HAk	100	IM	0	0.4±1.1	0.4±1.1	1.0±2.1	2.6±2.6	2.9±2.9	2.9±2.7	2.9±2.3	
5***	pCAGGS	100	IM	0	0	0	0	0	0	0	0	0

* Time of booster vaccination. ** GMT (log_2) ± SD. *** Data from the Chapter 5.

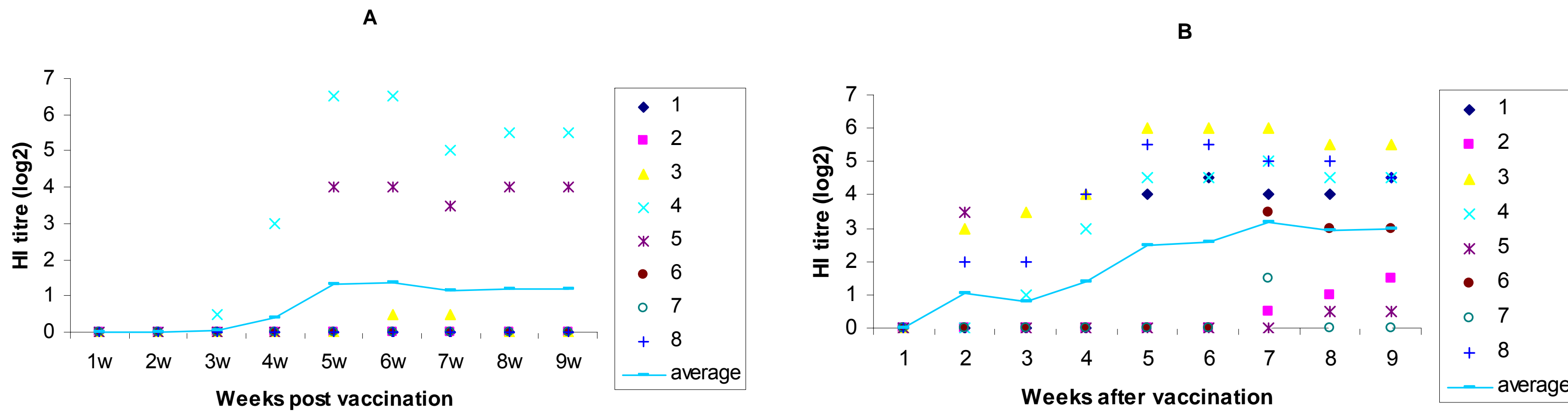

Figure 6.5 Temporal anti-H6 HI responses in chickens immunized with 10 µg (A) and 100 µg (B) pCAG-optiHAk construct. The curve represents the mean antibody titre of the relevant group. Each icon number refers to one bird.

6.3.4 Comparison of HI titres between pCAG-HAk and pCAG-optiHAk groups

The sero-conversion rate and level of antibody response were compared between pCAG-HAk and pCAG-optiHAk groups (Tables 6.2 and 6.3) using data from this experiment and from the experiment in Chapter 5. There were no significant differences in the sero-conversion rates from week 1 to week 7 after initial vaccination between either 10 or 100 µg groups for pCAG-HAk and pCAG-optiHAk vaccines. Neither were there significant differences (ANOVA or Mann-Whitney, $P>0.05$) during an 8-week period in antibody GMT post vaccination between pCAG-HAk and pCAG-optiHAk vaccines. Only at week 8 for the 10 µg dose groups was there a difference, with the pCAG-HAk group showing a higher sero-conversion rate than the pCAG-optiHAk group. This suggested that a codon-optimized HA-expressing DNA vaccine was not able to achieve better immunogenicity than the wild type HA-expressing DNA vaccine.

6.4 Discussion

The HA protein of AIV is a natural target for vaccines. In the study reported in this chapter, the virus HA gene sequence was replaced by the chicken biased codon sequence to prepare the vaccine. As a result, there was 25.2% alteration at the nucleotide level in the codon-optimized HA DNA sequence, but no changes at the amino acid level. The GC ratio increased 18.2% in the codon optimized HA gene compared to the wild type virus sequence. The high GC content in the gene may be favorable for its mRNA stability, processing and nucleo-cytoplasmic transport (Haas *et al.* 1996; Jiang *et al.* 2007). This high GC rate could increase the number of CpG motifs in an optimized gene, which may act as an inherent adjuvant. Theoretically, the immunogenicity of the optimized HA-

encoding plasmid could be expected to have been improved. However, the codon optimized HA DNA vaccine did not achieve better antigen expression in terms of band intensity in WB *in vitro*, or with immunogenicity in terms of sero-conversion rate and HI titre *in vivo*, than the wild type HA-expressing DNA vaccine.

The dose-response experiment showed that the 100 µg dose group elicited a better response than the 10 µg dose group and 87.5% birds vaccinated twice with a dose of 100 µg produced a mean HI titre of 2^3. Although the age of vaccinated birds differed for logistical reasons between the pCAG-optiHAk study (6-week-old chickens) and the pCAG-HAk study (3-4 week-old chickens), both groups were SPF chickens of the same strain and batch and the age difference was considered unlikely to affect their antibody responses.

Hass *et al.*(1996) reported that re-engineering the coding sequence with the most prevalent HIV-1 codons considerably impaired rat thymocyte protein (Thy-1) expression, but enhanced green fluorescent protein expression. Our experiment showed that either *in vitro* or *in vivo* expression of optimized HA gene was similar to that achieved by the wild type HA-expressing DNA vaccine. This result was different from other studies on optimized DNA vaccines, in which increased immunogenicity using a codon optimization strategy was shown (Deml *et al.* 2001; Jiang *et al.* 2007; Narum *et al.* 2001; Steinberg *et al.* 2005).

Many factors regulate and influence gene expression levels. Although others who employed the same strategy for codon optimization as used in this study achieved the expected increased immune response (Jiang *et al.* 2007; Steinberg *et al.* 2005), introduction of host biased codons into a DNA vaccine backbone alone may not be

enough to increase vaccine immunogenicity. Some factors such as mRNA secondary structure, internal TATA boxes, cryptic splicing sites, premature PolyA sites, internal chi sites, ribosomal binding sites, negative CpG islands, RNA instability motif (ARE) and inhibition sites all may impact on ribosomal binding and stability of mRNA (Wang *et al.* 2006). Thus, expression of a codon biased gene may still require further enhancement by RNA optimization.

In addition, it has been widely assumed that translational initiation is responsible for translational efficiencies of mammalian gene products (Haas *et al.* 1996). The toeprinting assay used with mRNAs showed that the nucleotide G in position +4 augmented recognition of AUG and favored translation (Kozak 1987b). The first amino acid following ATG was Glu (GAG) in an optimized HA gene from A/goose/Guangdong/1/96 (H5N1) (Jiang *et al.* 2007), in contrast, the current optimized HA gene presented Ile (ATC). The difference that the 4+ sequence was A rather than G in the current study may account for suboptimal expression in chicken cells.

Considering the factors above, the codon optimization approach in this study needs to be further explored.

Chapter 7

Effect of different expression vectors with the Kozak sequence on HA-expressing DNA vaccines against H6N2 avian influenza

7.1 Introduction

DNA-based immunization has pioneered a new era of vaccine research. Efficient expression of heterologous genes in an appropriate eukaryotic vector is a prerequisite for the development of DNA vaccines. A promoter/enhancer, responsible for transcriptional initiation, is critical to successfully drive the expression of heterologous genes of interest. Davis *et al*. (1993b) reported that luciferase reporter gene expression driven by the RSV promoter was 1,000-fold higher using the SV40 promoter. Direct DNA injection of a HIV-1 gag/env multigenic DNA vaccine under the CMV/IE promoter/enhancer generated stronger humoral and T-cell proliferative responses in macaques than those produced using the endogenous AKV murine leukaemia viral long terminal repeat (Galvin *et al.* 2000). The HA-expressing pCI-neo vector containing the CMV/IE promoter conferred better immune response to an H5 AIV in chickens in terms of antibody titre and survivability post virus challenge when compared with pSI containing the SV40 promoter, VR1012 containing CMV/IE promoter, pCAGGS.MCS containing the chicken β-actin promoter and pRC/RSV containing RSV promoter (Suarez and Schultz-Cherry 2000a). It is therefore important to select an effective vector with a strong promoter in an attempt to improve the efficacy of DNA vaccines.

Sequences flanking the AUG initiation codon within mRNA have been proven to impact on its recognition by eukaryotic ribosomes. The consensus sequence surrounding the start codon, known as the Kozak consensus sequence, GCCA/GCCAUGG, has been shown to

be critical for efficient translation because lack of these nucleotide bases caused leaky scanning of ribosomes and hence reduced the efficiency of translation (Kozak 2005). The insertion of a Kozak sequence adjacent to the target gene in a vector has been shown to significantly enhance the level of gene expression (Melkebeek *et al.* 2007; Olafsdottir *et al.* 2008).

In Chapters 3 and 4, a total of four eukaryotic vectors for expressing HA gene were investigated, however they did not elicit measurable HI antibody to H6 via the IM route in Hy-Line layer chickens. In Chapters 5 and 6, the pCAG-HAk and pCAG-optiHAk constructs generated using the pCAGGS vector, with the addition of a Kozak sequence, successfully induced measurable antibody responses in SPF chickens. Unfortunately, due to logistic reasons associated with the conduct of this work in Harbin, China, it was not possible to conduct homologous virus challenge. In view of the successful generation of H6 antibody with DNA vaccines using the pCAGGS expression vector and a Kozak sequence, experiments were conducted and reported in this chapter to determine whether the vector or the Kozak sequence played a more important role in antigen expression and generation of measurable antibody responses. It was believed that this might help to explain why the previous vectors did not induce detectable antibody responses.

7.2 Materials and Methods

7.2.1 Preparation of pCI-HAk DNA vaccine

The HA gene was subcloned from the pCAG-HAk vector into the pCI vector at the *Eco*RI and *Sma*I sites to generate a pCI-HAk construct, using the procedures described in section 4.2.1. The pCAG-HAk, pCAG-optiHAk, pCI-HAk, pCI-HA, VR-HA, pCI and VR1012 plasmid DNAs were prepared using the protocol described in section 3.2.3.

7.2.2 Comparison of protein expression by the DNA vaccine constructs

7.2.2.1 Preparation of primary chicken-embryo-fibroblast (CEF) cells

Primary CEF cells were prepared using standard procedures. The head, feet, wings and viscera of 10-day-old SPF chicken embryos were removed and the remaining embryo material was washed once with HBSS. After the embryos were minced with scissors, 2.5 mL of 0.25% trypsin was added and incubated at 37°C for 5 min. The digest was rinsed with 25 mL HBSS following removal of the supernatant. The cells were filtered through four layers of gauze, agitated and suspended in 30 mL DMEM with 6% FCS and then distributed into 100 mL flasks and incubated at 37°C until confluent. Primary cells were trypsinized, transferred to a 6-well plate as described in section 2.2.4.1 and cultured in DMEM supplemented with 5% FCS, 50 U/mL penicillin and 50 µg/mL streptomycin.

7.2.2.2 *In vitro* expression of DNA vaccine constructs

Chicken embryo fibroblast cells, human embryonic kidney cells (293T), African green monkey kidney cells (Cos-7) and hamster kidney cells (BSR) were grown in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine and 10% FCS as described in section 2.2.4. Transfection was performed as described in sections 4.2.2 and 5.2.3; expressed H6 HA protein was visualized by WB as described in section 4.2.3 and by IFT as described in section 2.2.5.

7.2.3 Immunization of chickens

Three-week old Hy-Line layer chickens were housed in free range animal house pens, with access to water and feed *ad libitum*. They were randomly assigned into three groups with five birds each and a naive control group with six birds. The three vaccinated groups

were vaccinated twice at 3-week intervals with the pCAG-HAk, pCAG-optiHAk or pCI-HAk vaccine constructs using the same immunization procedure as in section 4.2.3.

7.2.4 Virus challenge and virus isolation

Three weeks following booster vaccination, each bird was subjected to challenge using a 0.5 mL H6N2 virus challenge dose ($10^{6.5}$ EID_{50} /0.1 mL) as described in section 2.2.8. OS and CS were collected every second day over a seven-day period. Virus isolation was performed as described in section 2.2.9.

7.3 Results

7.3.1 Confirmation of the pCI-HAk DNA vaccine construct

The pCI-HAk construct demonstrated the expected size fragments following restriction endonuclease digestion, as illustrated in Figure 7.1. These were 1.7 kb and 4.0 kb fragments after *Eco*RI and *Xma*I digestion and 2.0 kb and 3.7 kb fragments after *Eco*RI and *Bam*HI digestion. This construct was further confirmed by sequencing the insert gene and its junction site (data not shown).

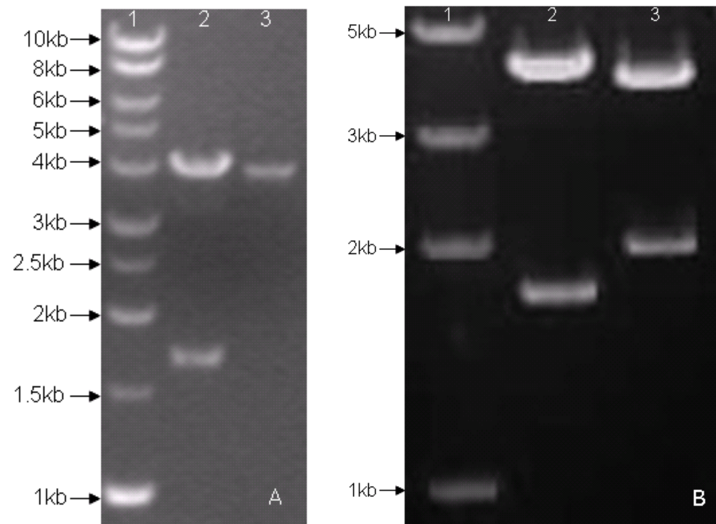

Figure 7.1 Restriction enzyme analysis of the plasmid pCI-HAk.
A Lane 1, 1 kb DNA ladder (Promega); lane 2, pCI-HAk (*Eco*RI/*Xma*I); lane 3, pCI (*Eco*RI).
B Lane 1, DL2000 plus marker (Beijing Tuoyingfang Biotech Co., Ltd); lane 2, pCI-HAk (*Eco*RI/*Xma*I); lane 3, pCI-HAk (*Eco*RI/*Bam*HI).

7.3.2 Transient expression of different DNA vaccine constructs *in vitro*

7.3.2.1 Expression in chicken-origin cells

As shown in Figure 7.2, the pCAG-HAk and pCAG-optiHAk constructs showed similar intensity of fluorescence, but much better expression in terms of number of staining transfected cells, than the pCI-HAk construct. The pCI-HAk construct gave much brighter fluorescence and better expression in terms of number of transfected cells, than the pCI-HA construct. No specific fluorescence was observed in the blank vector, pCAGGS or pCI transfected CEF cells.

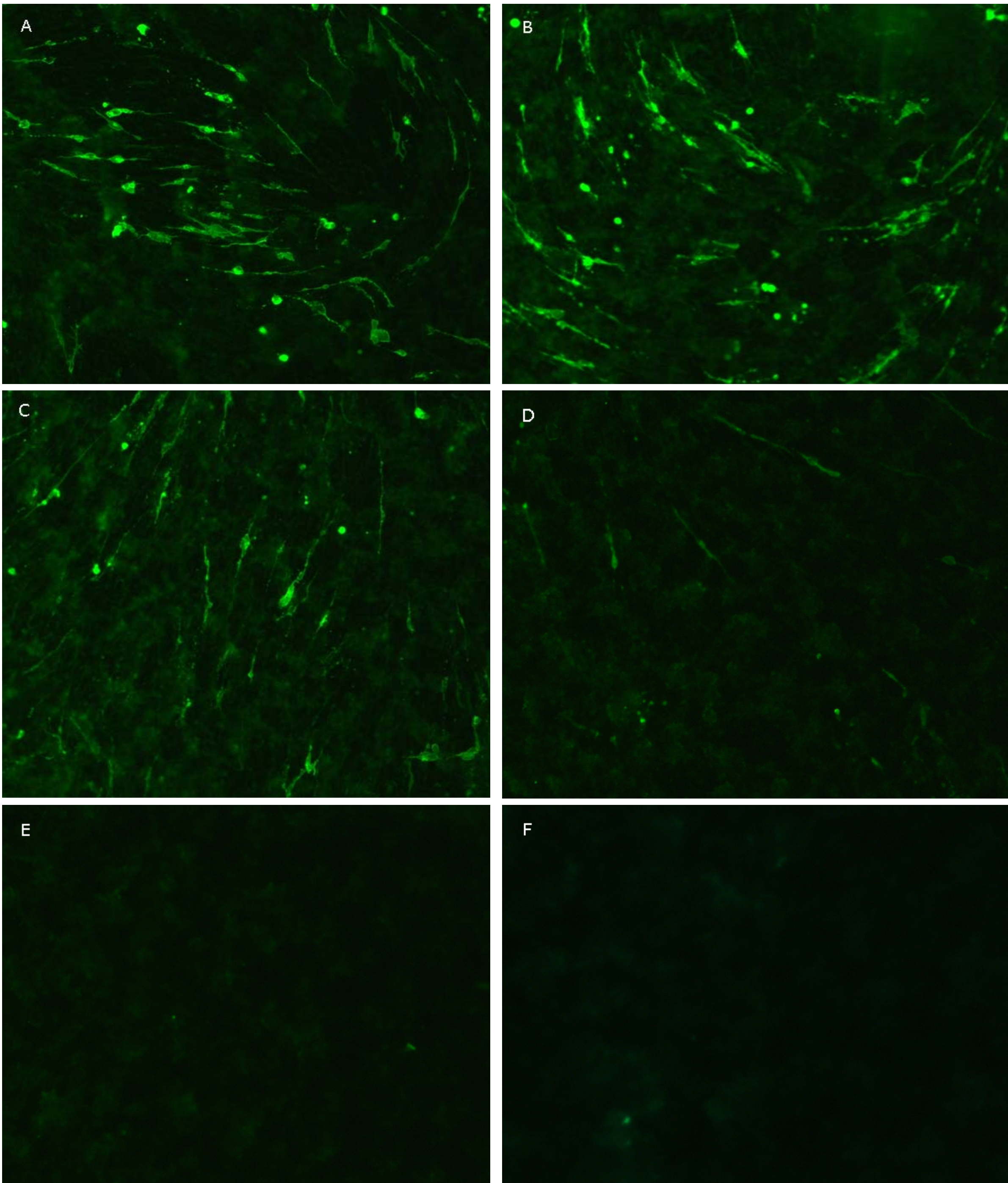

Figure 7.2 Photomicropgraphs of immunofluorescent staining for H6 avian influenza antigens in CEF cells transfected with (A) pCAG-optiHAk, (B) pCAG-HAk, (C) pCI-HAk ,(D) pCI-HA, (E) pCAGGS only, (F) pCI only. (Leica DMIRES2, x100).

7.3.2.2 Expression in human-origin cells

Similar to the observations in chicken origin cells, the brightest H6 antigen immunofluorescence was present in 239T cells transfected with the pCAG-HAk and pCAG-optiHAk constructs, followed by the pCI-HAk and pCI-HA constructs. The pCAGGS or pCI transfected cells did not show specific fluorescence (Figure 7.3).

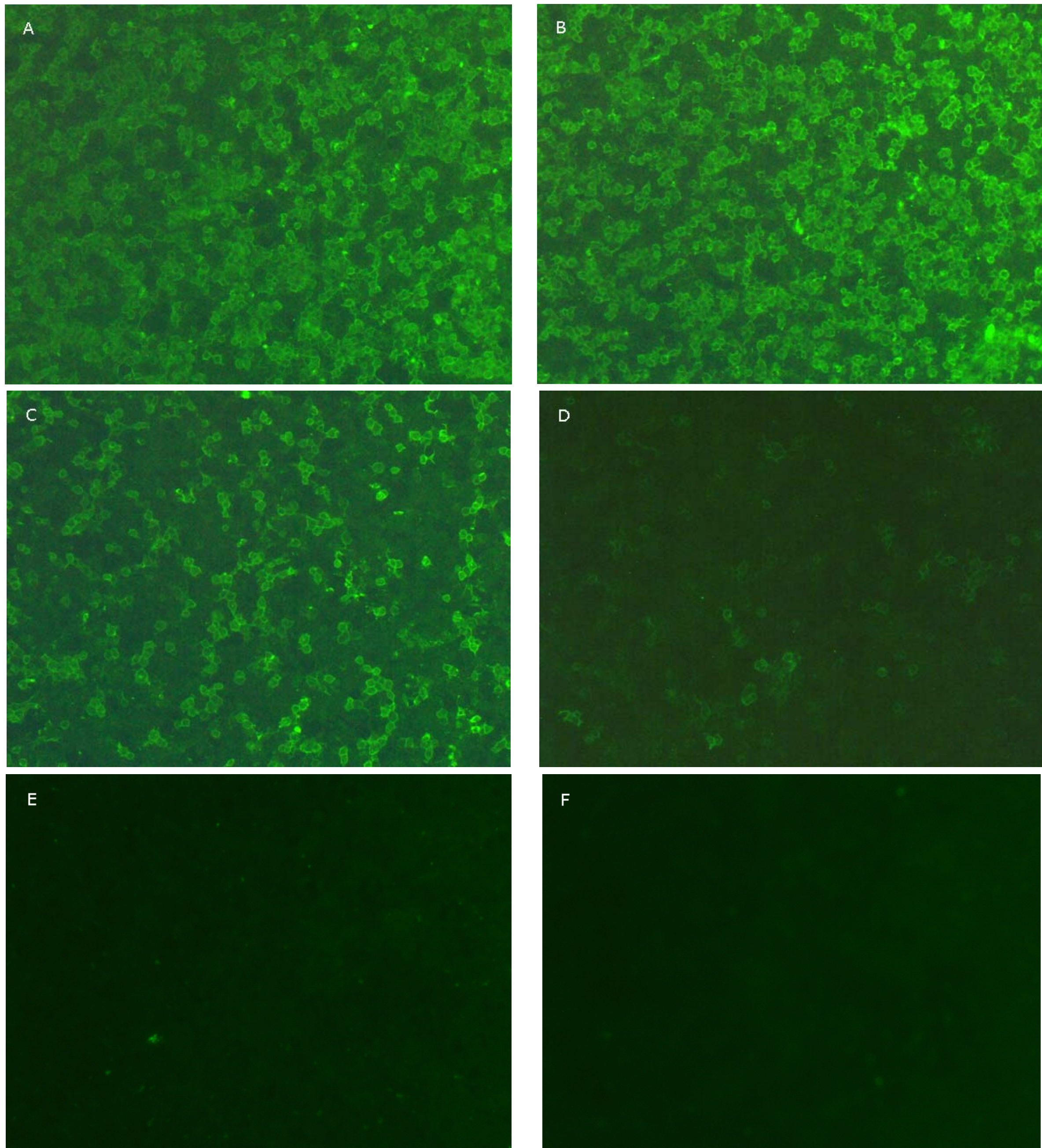

Figure 7.3 Photomicropgraphs of immunofluorescent staining for H6 avian influenza antigens in 293T cells transfected with (A) pCAG-optiHAk, (B) pCAG-HAk , (C) pCI-HAk, (D) pCI-HA, (E) pCAGGS only, (F) pCI only. (Leica DMIRES2, x100).

7.3.2.3 Expression in monkey-origin cells

Cos-7 cells were transfected with 5 µg of the different DNA constructs, as described in

section 3.2.3, using FuGENE® HD Transfection reagent (Roche). At 48 hr following transfection the transfected cells were lysed in 100 µL non-reducing buffer. Aliquots of 5 µL of lysed cells for each of the pCI-HAk, pCAG-HAk and pCAG-optiHA constructs and 20 µL transfected cells for each of the other plasmids (pCI-HA, VR-HA, pCI, pCAGGS, VR1012) were loaded in their respective lanes for SDS-PAGE as described in section 3.2.5.3.

Western blotting was conducted and gels were probed with antibody to H6 influenza virus. As shown in Figure 7.4 (A), the pCAG-HAk and pCAG-optiHA constructs gave the strongest bands. Next was the pCI-HAk construct. These three constructs were at least 4-fold stronger in band intensity than either the pCI-HA or VR1012-HA constructs and the band intensity for VR-HA was clearly stronger than that for pCI-HA.

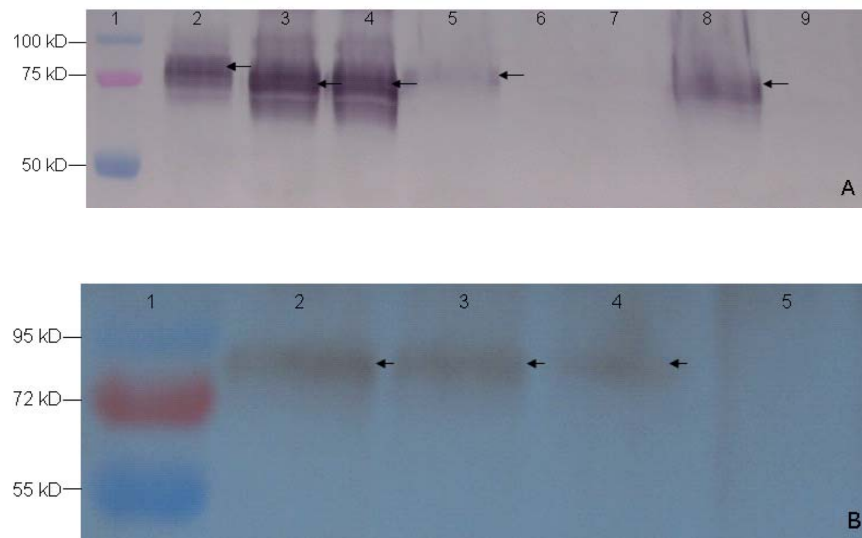

Figure 7.4 (A) HA protein expression in DNA vaccine transfected Cos 7 cells by Western blotting. Lane 1, marker (Bio-Rad); lane 2, pCI-HAk; lane 3, pCAG-HAk; lane 4, pCAG-optiHAk; lane 5, pCI-HA; lane 6, pCI; lane 7, pCAGGS; lane 8, VR-HA; lane 9, VR1012. **(B) HA protein expression in transfected BSR cells by Western blotting.** Lane 1, marker (Fermentas); lane 2, pCAG-optiHAk; lane 3, pCAG-HAk; lane 4, pCI-HAk; lane 5, pCAGGS.

Also, as shown in Figure 7.5, the intensity of H6 immunofluorescence and the level of expression in terms of number of transfected Cos-7 cells appeared to be marginally stronger for pCAG-HAk than for pCI-HAk, but for the pCI-HA and VR-HA constructs the intensity appeared similar. None of the negative control groups showed specific bands or immunofluorescence.

7.3.2.4 Expression in hamster-origin cells

Similarly to the outcome in the Cos-7 cells with WB, the expression of pCAG-optiHA and pCAG-HA constructs in BSR cells gave similar intensity bands, which were denser than those from the pCI-HAk construct (Figure 7.4, B).

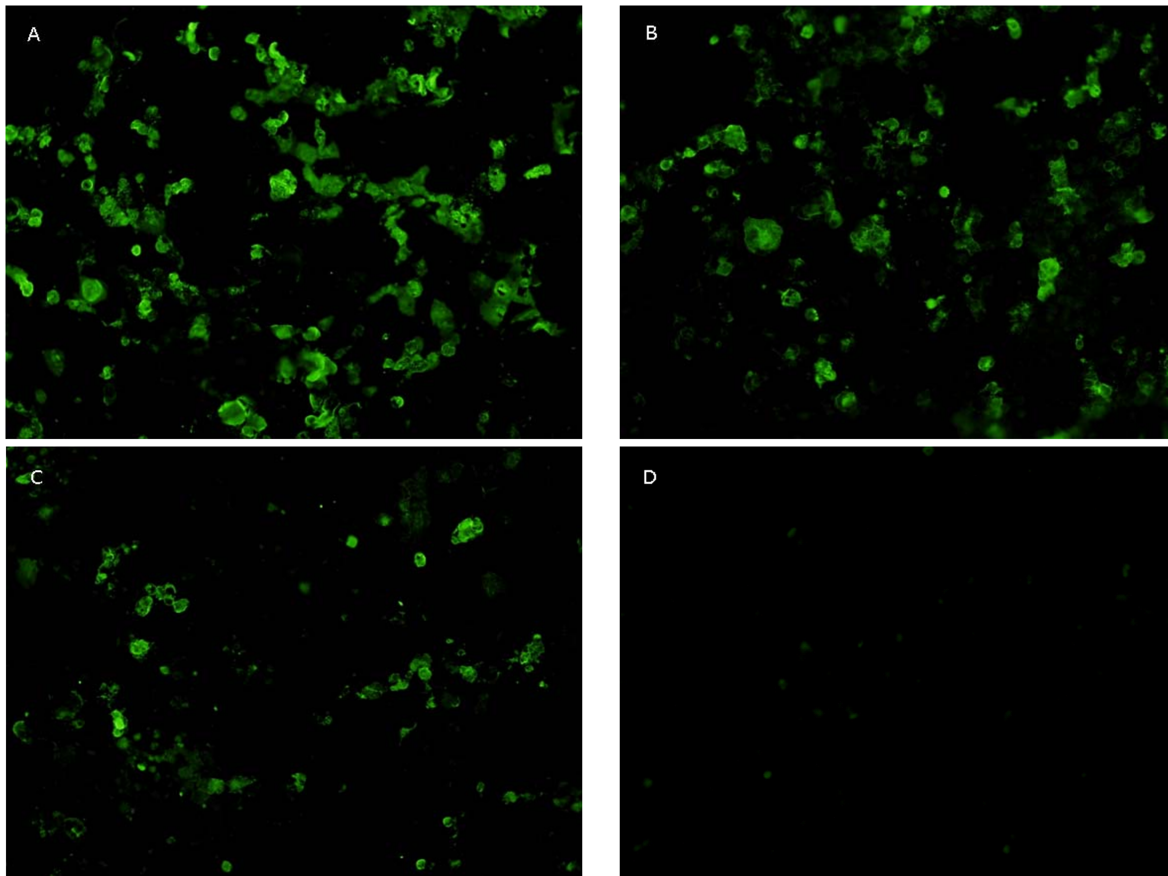

Figure 7.5 Photomicrographs of immunofluorescent staining of transfected Cos-7 cells with (A) pCAG-HAk, (B) pCI-HAk, (C) VR-HA, (D) Cos-7 cells. (Olympus BX51, x10).

7.3.3 Comparison of immune responses following administration of the pCI-HAk, pCAG-HAk and pCAG-optiHAk constructs in chickens

As indicated in Table 7.1, no antibody production was measurable in Hy-line chickens vaccinated twice with the 100 µg pCI-HAk, pCAG-HAk or pCAG-optiHAk plasmids. However, all three vaccine groups and the naive control group showed a significant increase in GMT of HI antibody (P= 0.001) after challenge using the paired-sample T test. Although the HI GMT post-challenge in the pCI-HAk group was higher than the other two vaccinated groups, there were no significant differences in GMT between the pCI-HAk, pCAG-HAk or pCAG-optiHAk groups and the naive control group. Similarly there were no significant differences in HI GMT post challenge between the pCAG-HAk, pCAG-optiHA or pCI-HAk groups.

Table7.1 Antibody response prior to and post virus challenge.

	Naïve control	pCAG-HAk	pCAG-optiHA	pCI-HAk
Prior to challenge	0	0	0	0
Post challenge	6.0±2.0*	5.6±1.7	5.0±3.4	8.2±2.2

* Values represent GMT (log_2) ± SD of each group.

As shown in Table 7.2, there appeared to be a reduction in virus recovery from OS and CS in all three vaccinated groups compared with the naive control group. However, the differences in the proportion of OS or CS positive for H6N2 virus between the naive control group and the pCAG-HAk, pCAG-optiHA or pCI-HAk vaccinated groups were not statistically significant.

In terms of percentage of virus recovery from OS and CS, the pCAG-HAk group was lower than the pCAG-optiHAk or the pCI-HAk groups although there were no significant differences.

Table 7.2 Frequency of virus shedding in oropharyngeal and cloacal swabs.

	Naïve control	pCAG-HAk	pCAG-optiHA	pCI-HAk
OS	17/24(70.8)*	9/20(45)	12/20(60)	12/20(60)
CS	3/24(12.5)	0/20(0)	0/20(0)	1/20(5)

* No. of swabs positive for virus isolation/total number of swabs tested. Percentages are shown in parentheses.

7.4 Discussion

7.4.1 Comparison of *in vitro* expression for different HA-expressing constructs

The *in vitro* studies reported in this chapter demonstrated that H6 HA gene expression in mammalian and avian cells could be maximally enhanced by use of an optimum expression vector, in this case pCAGGS, plus the use of a Kozak sequence in the construct. Additionally, the *in vitro* expression of the H6 HA gene using the expression vector pCI-HA, shown to be of lower efficiency in Chapter 4, was substantially enhanced by addition of a Kozak sequence to the construct.

Four types of cells (monkey, hamster, human and chicken) were used to examine the *in vitro* HA expression of the DNA expression vectors by IFT and WB in the transfection experiments in this chapter. When the results are considered in conjunction with the *in vitro* comparisons in Chapter 4, the transfection experiments show a hierarchy in the *in vitro* expression efficiency in the order of pCAG-optiHAk/ pCAG-HAk > pCI-HAk > VR-HA > pCI-HA > pCI-neo-HA > pVAX-HA.

DNA vaccines usually consist of bacterial plasmids that contain heterologous genes under the control of a eukaryotic promoter (Garmory *et al.* 2003). The selection of a promoter/enhancer element depends upon both the target cell type and the functional design of the vector construct (Harms and Splitter 1995). Furthermore, the

polyadenylation sequence used in a vector backbone, responsible for transcriptional termination, may also have a significant effect on foreign gene expression (Garmory *et al.* 2003). In the pCAGGS vector, the CMV enhancer and the chicken β-actin promoter sequences are located upstream of the multiple cloning site (MCS) region and a rabbit β-globin poly (A) sequence is located downstream from the MCS region (Galbiati *et al.* 2000). In contrast, the pCI expression vector contains the CMV/IE enhancer/promoter region, a β-globin/IgG chimeric intron and an SV40 late polyadenylation signal. The VR1012 vector carries a CMV/IE promoter, a CMV intron A enhancer and a bovine growth hormone (BGH) polyadenylation sequence. These differences in vector backbones between different expression vectors may explain why the pCAGGS vector had slightly stronger expression of the same HA gene *in vitro* than the pCI vector, and why the VR1012 vector showed better *in vitro* expression than the pCI vector.

7.4.2 Effect of Kozak sequence on *in vitro* expression

As illustrated in Figure 7.5, the difference in band intensity of expression between the pCI-HAk and the pCI-HA (Kozak effect) was much greater than that between the pCAG-HAk and pCI-HAk (vector effect). This indicates that the Kozak sequence appeared to play a more important role in initiating protein expression among three efficient DNA vectors than the vector effect. It highlighted that the Kozak sequence can be used as an effective tool to increase the expression of target genes for DNA vaccine design in chickens. *In vitro* expression experiments with the pCAG-HAk vector also demonstrated that both the Kozak sequence and the pCAGGS expression vector were accountable for the increased level of expression, suggesting that both had a synergistic effect on the expression of DNA constructs. It also implies that mechanisms of improved antigen

expression for a Kozak sequence and modified vectors are different. Therefore, expression vector optimization and insertion of a Kozak sequence could synergistically enhance the expression of the gene of interest *in vitro* and hence potentially increase the efficacy of DNA vaccines in chickens.

7.4.3 Comparison of DNA construct-induced immunity in chickens

With all three vaccine constructs tested there appeared to be a reduction in virus shedding post-challenge compared to naive control chickens. Notably, with the pCAG-HA and pCAG-optiHA constructs there was no cloacal shedding of H6 virus. However, with the small group sizes and individual bird variation to DNA vaccination these differences were not shown to be significantly different (P>0.05) in contrast to the results with some of the vaccine constructs reported in Chapters 3 and 4 where similar reductions in virus shedding were observed and in some cases were statistically significant. The reduction in virus shedding via cloaca or oropharynx in the vaccinated chickens showed a hierarchy: pCAG-HAk > pCAG-optiHAk > pCI-HAk, which was similar to the hierarchy of gene expression *in vitro*.

DNA vaccines have been demonstrated to elicit antibody responses since their first description in 1990 (Wolff *et al.* 1990), but their capacity to induce antibody responses has been shown to be quite variable in chickens (Chen *et al.* 2001; Kodihalli *et al.* 1997). In a previous study, chickens vaccinated with an H5 AI DNA vaccine by GG did not produce detectable pre-challenge antibodies, but produced high antibody titre and provided complete protection after homologous virus challenge (Kodihalli *et al.* 1997). Our results showed similarities with that study although the challenge was with LPAI virus rather than HPAI virus and we used reduction in virus shedding as our indicator of

protection. Using the less efficient delivery system of IM injection, the pCAG-HAk and pCAG-optiHAk vaccines did not induce measurable antibody before challenge in Hy-Line chickens, but showed a reduction in oropharyngeal shedding and absence of cloacal shedding after challenge. Evidence of protection after DNA vaccination in the absence of humoral immunity is considered an indicator of cellular immunity (Kodihalli *et al.* 1997).

Examination of the impact of expression vectors on immune responses has generally compared only two plasmid configurations (Barry and Johnston 1997; Davis *et al.* 1993b; Galvin *et al.* 2000; Melkebeek *et al.* 2007; Uchijima *et al.* 1998; van Drunen Littel-van den Hurk *et al.* 1999; Xiang *et al.* 1995) or three plasmid vectors (Miller *et al.* 2004). Little has been reported on the comparison of multiple expression vectors (Suarez and Schultz-Cherry 2000a). Five expression vectors containing different combinations of the widely used CMV promoter, the chicken β-actin promoter and various enhancer and polyadenylation sequences were investigated to provide useful information on the selection of an appropriate vector for the development of a DNA vaccine in chickens.

7.4.4 Analysis of the absence of detectable antibody responses using these DNA vaccines

In the vaccination and challenge study conducted with the three vaccine constructs (pCAG-HAk, pCAG-optiHAk and pCI-HAk) in Hy-Line chickens, none of the constructs induced a H6 HI antibody response prior to challenge. This was in marked contrast to the results reported in Chapters 5 and 6 where both pCAG-HAk and pCAG-optiHAk constructs elicited measurable HI antibody responses in intramuscularly vaccinated SPF (White Rock) chickens. The reasons for this unexpected result are unclear, but possible reasons are considered below and highlight potential areas for future investigation.

Although the mechanisms of induction of immunity by DNA vaccines remain poorly understood, it is accepted that DNA vaccines are more effective in producing protective immunity in mice than in larger animals and non-human primates (Hirao *et al.* 2008). Some studies showed that the efficiency of responses to plasmid DNAs was highly related to the genotype of mice (Barry and Johnston 1997; Doolan *et al.* 1996). For example, immunization of different strains of mouse with plasmid DNAs encoding influenza virus HA, NA and NP genes showed that the ELISA antibody titres to HA or NA molecules in BALB/c, C3H and B10 mice were high, intermediate and low, respectively. HA-DNA afforded effective protection against lethal virus challenge only in BALB/c mice, whereas, NA-DNA provided significant protection in all strains of mouse. NP-DNA failed to provide protection in any strain of mouse (Chen *et al.* 1999b). This genetic control of antibody response among mouse strains was also observed using influenza virus HA subunit vaccines (Hirabayashi *et al.* 1991). Furthermore, considerable variation in antiviral antibody titres occurs in humans who received a conventional inactivated influenza vaccine and this may be partly explained by genetic factors (Kasel *et al.* 1969). In the current studies, since two chicken breeds were used for evaluating the potency of the same vaccine in two separate experiments, and one breed gave no post-vaccinal antibody response, one can speculate that chicken breed differences have an effect on antibody responsiveness to DNA vaccines. This will need to be further investigated.

Another point to consider is that there were differences between the rations fed to the Hy-Line chickens at Murdoch University and the White Rock SPF chickens at Harbin. The poultry rations fed to the Hy-Line chickens contained approximately 10% lupin seed

(*Lupinus angustifolius*) as a protein additive (Brenes *et al.* 1993). Australian sweet lupins are widely used as a source of protein and energy in livestock feeds. Some experimental studies have indicated that feed containing lupins was shown to lower or inhibit antibody responses to experimental subunit vaccines to spirochaetosis in chickens and pigs (Dr Song Yong, personal communication). This did not however appear to affect antibody response to the inactivated whole virus H6N2 vaccines used in Chapters 3 and 4. Whether the presence of lupins in the ration affected the antibody responsiveness to DNA vaccines in chickens needs further investigation.

An additional factor differed in the pCAG-HAk and pCAG-optiHAk vaccination studies undertaken at Murdoch University and Harbin. This was the method for extraction of the plasmid DNA from the bacterial suspension to prepare a large quantity of plasmid DNA for the vaccine. The alkaline lysis plus PEG method (section 5.2.2) was used to prepare plasmid DNA for the SPF chicken vaccination, whereas, the modified simple alkaline lysis method (section 3.2.3) was used to prepare plasmid DNA for Hy-Line chicken immunization. This was due to the different protocols and the reagent availability in the two laboratories. The different methods used may have resulted in variations in the quality of the plasmid DNA, which in turn could affect the efficacy of DNA vaccines *in vivo,* even though expression of plasmid DNA prepared from the modified alkaline lysis method was successfully demonstrated in Cos-7 cells. The role of different extraction methods for HA-expressing DNA vaccines in Hy-Line chickens also needs further investigation.

Other mechanisms, not identified in these studies, may also have contributed to the difference in antibody responses between the Hy-Line and SPF chickens.

7.4.5 Correlation between *in vitro* and *in vivo* expression

Higher levels of antigen expression *in vitro* may not necessarily mean higher levels of protein expression *in vivo* and also the induction of immune responses depends on the induction of cytokines such as interferon and interleukin which may result from ISS in the plasmid DNA (Sato *et al.* 1996). Nevertheless, analysis of some DNA vaccination studies indicates that there was a direct correlation between the level of protein expression *in vitro* and immunogenicity in mice (Steinberg *et al.* 2005), pigs (Melkebeek *et al.* 2007) and chickens (Jiang *et al.* 2007; Suarez and Schultz-Cherry 2000a). In the present study, the pCAG-HAk construct showed a similar level of expression in mammalian cells to the pCAG-optiHAk construct and both constructs also induced similar antibody responses in SPF chickens (as described in Chapter 6) and gave a similar level of reduction in virus shedding in the current trial. There was also a correlation between *in vitro* and *in vivo* expression when comparing the pCAG-HAk with the pCI-HAk construct. Interestingly, our results indicated that the pCAGGS construct was most efficient among the five DNA vectors evaluated, although the pCAG-HAk construct introduced two varaibles (i.e. a vector with different promotor and a Kozak sequence) at the same time. In other studies the pCI-neo vector induced the strongest H5 antigen expression in mink lung epithelial cells and CEF cells among five vectors (pCAGGS.MCS, VR1012, pCI-neo, pSI, pRC/RSV) and also provided the best antibody responses to H5 HA in chickens (Suarez and Schultz-Cherry 2000a). However, the current pCAGGS vector used in this study was possibly further modified from the pCAGGS.MCS backbone used in that study and the inserted HA gene was different between the two studies.

Overall it is considered that the level of protein expression following transfection into mammalian or avian cells by the DNA plasmid vaccines evaluated shows a direct relationship with the magnitude of immune responses in chickens receiving these DNA vaccines. Since the level of *in vitro* expression correlates with the level of immune response *in vivo*, it is also considered that *in vitro* expression levels of the plasmid vaccines can be used as an indicator for pre-selection of expression plasmids prior to development of a DNA vaccine. A separate issue that has also been highlighted in these studies is that even with the optimum vector and promoters from *in vitro* assessment of a plasmid construct, it will be necessary to optimize the factors leading to effective *in vivo* expression of the antigen in order to initiate immune responses in different breeds of chickens or other target species.

Chapter 8

Evaluation of different chemical adjuvants on avian influenza DNA vaccines in chickens

8.1 Introduction

Rapid degradation and poor cellular uptake of plasmid DNA has significantly impacted on the efficacy of 'naked' plasmid DNA vaccines (Wilson *et al.* 2009). As adjuvants have historically played a major role in successful vaccine development (Scheerlinck *et al.* 2006), a number of chemical adjuvants have been examined in an attempt to improve plasmid DNA immunogenicity. Conventional adjuvants, many of which are very effective for killed and subunit vaccines, have either not been tested for DNA vaccines or are not usually beneficial in the mouse model (van Drunen Littel-van den Hurk *et al.* 2004). However, some studies have shown a beneficial effect with such adjuvants (Jin *et al.* 2004; Sasaki *et al.* 1998a; Sasaki *et al.* 1998b; Sasaki *et al.* 1998c). Thus, further investigation of conventional or novel adjuvants and their formulation technologies could improve the utility of plasmid DNA vaccination.

Aluminum salts, referred to as alum, including aluminum phosphate, aluminum hydroxide and other aluminum-containing salts, have been extensively characterized and are commonly used as conventional adjuvants to increase the magnitude of humoral immune responses. They are currently licensed for human and animal use (Gupta 1998). However, their potential as DNA vaccine adjuvants has had limited investigation. Alum-DNA formulations increased the capacity of some DNA vaccines to induce antibody responses up to 100-fold in mice and guinea pigs, and 5-10-fold in non-human primates (Ulmer *et al.* 1999). Also, the level of antibodies induced by a plasmid DNA-coated

PLG-CTABmicro-particles in mice was markedly enhanced by the addition of aluminum phosphate (Singh *et al.* 2000).

Nano-particles, varying in size from 10 to 500nm, are solid particles made from inert materials (Aucouturier *et al.* 2001). They are potentially biocompatible and biodegradable, are relatively stable *in vivo*, are relatively easy to link with immunogens, have straightforward delivery methods and show little or no side effects (Cui and Mumper 2002a; Scheerlinck and Greenwood 2006). As early as 1995, solid inert beads adsorbed with antigen were reported to prime $CD8^{+}$T cell responses (Falo *et al.* 1995). Inert nanoparticles have subsequently been shown to induce strong immune responses to protein and peptide antigens in mice (Fifis *et al.* 2004a; Fifis *et al.* 2004b), sheep (Scheerlinck *et al.* 2006), pigs (Aucouturier *et al.* 2001) and cattle (Aucouturier *et al.* 2001). In the context of DNA vaccines, a cationic nanoparticle formulated plasmid DNA encoding a reporter gene enhanced *in vitro* cell transfection efficiency and elicited 16-200-fold greater immune responses in mice than naked plasmid DNA alone following multiple delivery routes (Cui and Mumper 2002a, b, c, 2003a). Co-administration of cholera toxin (CT) and lipid A with a nanoparticle-based plasmid DNA showed a synergistic effect and hence further enhanced immune responses (Cui and Mumper 2003a). Thus, nanoparticles, as a novel class of adjuvants, have the potential to induce immune responses to protein or plasmid DNA immunogens without the side effects typically associated with local tissue damage caused by conventional chemical adjuvants. Though the microparticle formulated plasmid DNA encoding the NP gene of A/PR/8/34 (H1N1) virus was shown to enhance immune response in mice (Hartikka *et al.* 2008),

there has been no reports on nanoparticle-based avian influenza DNA vaccines in chickens.

Seppic (Paris, France) produces a well-known range of adjuvants, known as Montanide ISA series, for human and veterinary vaccines. For example, foot and mouth disease (FMD) recombinant protein adjuvanted with Montanide-ISA 50V elicited an FMD-specific humoral immune response and provided protective immunity in guinea pigs (Balamurugan *et al.* 2005). Most recently the company has developed and provided a polymeric adjuvant, Essai 849101 for DNA vaccination.

Genetic control of antibody responses have been observed in mice (Barry and Johnston 1997; Chen *et al.* 1999b; Doolan *et al.* 1996; Hirabayashi *et al.* 1991) and probably in humans (Kasel *et al.* 1969). However, co-administration of cholera toxin B subunit (as adjuvant) with influenza HA vaccine not only markedly augmented the antibody responses to HA in all mouse strains tested, but also the degree of enhancement was similar among the strains (Hirabayashi *et al.* 1991). In Chapter 7, Hy-Line chickens receiving the pCAG-HAk vaccine construct did not produce a measurable antibody response in contrast to White Rock SPF chickens which did produce a measurable response to this vaccine (Chapters 5 and 6). Therefore, it was also of interest to determine whether the use of adjuvants could modify this possible genetic effect on antibody response to the same DNA vaccine.

In Chapter 3 the chemical adjuvant lipofectin was evaluated in chickens and showed some enhancement of the immune response in terms of increasing the seroconversion rate of vaccinated birds and the level of antibody production and reducing virus shedding in both oropharyngeal and cloacal swabs. However, it is prohibitively expensive for routine

use in poultry. Thus, an experimental adjuvant for poultry DNA vaccines (Essai), a new nanoparticle (Phema) and two variations of conventional alum were investigated as adjuvants with a plasmid DNA vaccine in chickens and the results are reported and discussed in this Chapter.

8.2 Materials and Methods

8.2.1 Plasmid DNA used for the vaccine

The pCAG-HAk plasmid DNA used as the vaccine for combination with the various adjuvants was prepared as described in section 3.2.3.

8.2.2 Formulation of plasmid DNA vaccines with adjuvants

8.2.2.1 Preparation of DNA-coated alum

Aluminum hydroxide ($Al(OH)_3$) (AH) gel was prepared from aluminum potassium sulphate ($AlK(SO_4)_2.12H_2O$) (BDH Chemicals Ltd, Victoria, Australia) as described by Scheerlinck *et al.* (2006). Briefly, 10 mL of 10% aluminium potassium sulphate was added to a 50 mL conical tube then 22.8 mL of 0.25N NaOH (BDH) was added drop-wise while vortexing. Following incubation for 10 min at room temperature, the precipitate was collected by centrifugation at 1,000 g for 10 min. Following removal of the supernatant, 50 mL dH_2O was added to re-suspend the AH gel by vortexing. The gel was washed once in 50 ml of water followed by centrifugation at 900 g for 20 min. Two versions of the AH adjuvant gels were prepared at a concentration of 400 μg/μL. One was suspended in 0.9% NaCl (referred to as alum-NaCl) and the other in PBS (referred to as alum-PBS). Then 1mL of the 400 μg/μL AH suspensions was mixed with equal volumes of 0.9% NaCl or PBS solutions containing 0.2mL plasmid DNA (9.3 μg/μL)

respectively. These were incubated for the various times and temperatures indicated below, followed by centrifugation at 3,000 g for 10 min at room temperature. The DNA concentration in the supernatant was measured using a Nanodrop spectrophotometer. The precipitate was dissolved in either 0.9% NaCl or PBS respectively. The amount of bound DNA in the precipitate was calculated by subtracting the amount of DNA in the supernatant from the total DNA used to prepare the suspension. The emulsions were then diluted to a concentration of 0.5 μg/μL DNA in their respective solutions for direct IM injection.

To obtain the optimal DNA-binding to the alum adjuvants, different concentrations of alum (400, 200, 100, 50 μg/μL), different diluting buffers (PBS or 0.9% NaCl), different binding times (0.5, 1, 3, 5, 8, 27 hr) and different temperatures (4°C and 22°C) were used to prepare the adjuvants, using a constant concentration of plasmid DNA (2.4 μg/μL).

8.2.2.2 Preparation of DNA-coated Essai

Essai 849101, a high molecular weight water soluble copolymer, was kindly provided by Seppic, France. Formulation of the plasmid DNA using Essai 849101 was carried out according to the manufacturer's instructions. Briefly, DNA was diluted in PBS and then Essai was added dropwise with gentle stirring to the DNA preparation to give final concentrations of 0.5 μg/μL for DNA and 10% (v/v) for Essai. Following initial mixing, the mixture was stirred for a further 10 min at room temperature.

8.2.2.3 Preparation of the DNA-conjugated nano-beads

A novel polymer (2-hydroxyethyl-methacrylate) (Phema), which formed a 150-300 nm hydrophilic nanoparticle suspension, was provided by the Nanotechnology Group,

Murdoch University. The DNA-coated nano-beads were prepared as follows. Optimization of the solvent for mixing the Phema and DNA was determined using 100% dH_2O, dH_2O/ethanol (50:50) and 100% ethanol and calculation of the level of DNA binding. Consequently 2 mL of 1% Phema (w/v in ethanol) was added into chilled 10 mL tubes on ice and sonicated for a few seconds. Then 1 mL of 1% polyvinyl alcohol (PVA) (w/v in dH_2O) was added and the mixture was sonicated for 1 min on ice. Subsequently 200 µL of plasmid DNA (9.3 µg/µL) was added and sonicated for 2 min on ice. The resultant solution was then filtered through a 0.2 µm membrane to remove the surfactant. The flow-through was centrifuged at 15,000 g for 20 min at room temperature. The pellet was dissolved in PBS and the amount of DNA present was calculated by subtracting the amount of DNA in the supernatant, measured by a Nanodrop spectrophotometer, from the total DNA added.

8.2.3 Vaccination regime used in chickens

Three-week old Hy-Line chickens were housed in free-range pens with access to water and feed *ad libitum*. The experimental protocol used is shown in Table 8.1. Birds received two 0.2 mL IM injections of each vaccine combination at 3 week intervals, with 0.1 mL per dose in each leg.

Table 8.1 Immunization regime used in chickens.

Group	Vaccine type	Dose (µg)	Adjuvant	No. of birds
1	pCAG-HAk	100	No	5
2	pCAG-HAk	100	alum-NaCl	5
3	pCAG-HAk	100	alum-PBS	5
4	pCAG-HAk	100	Essai	5
5	pCAG-HAk	200	Phema	4
6	pCAG-HAk	100	Phema	3
7	pCAG-HAk	10	Phema	5
8	PBS	100	No	6

8.2.4 Virus challenge and virus isolation

Three weeks following the booster vaccination, all birds in each group were subjected to virus challenge. Subsequently, OS and CS were collected every second day for virus isolation as described in section 7.2.4.

8.3 Results

8.3.1 Optimization of DNA vaccine adjuvant formulations

As shown in Table 8.2, the DNA binding rate increased with the increase of alum-NaCl concentration. DNA binding with alum-NaCl occurred within the first 1 hr. Temperature did not affect DNA binding rate after 3 hr. The 400 µg/µL alum-NaCl absorbed nearly 100% plasmid DNA at either room temperature or 4°C after 1 hr following mixing. Thus, 400 µg/µL alum-NaCl and binding for 2 hr at room temperature was used for the formulation of the DNA vaccine. In contrast, alum-PBS did not bind plasmid DNA even after overnight incubation.

As shown in Table 8.4, Phema adjuvant prepared with 100% ethanol as solvent gave the highest DNA binding rate. Thus, 100% alcohol was used for the preparation of the Phema adjuvant vaccine. Subsequently, the maximal plasmid DNA-Phema binding rate, determined using a constant Phema concentration and different DNA concentrations, was approximately 30%.

Table 8.2 Plasmid DNA concentration remaining in supernatant after plasmid DNA binding with alum/0.9%NaCl diluent.

Time	0.5 hr		1 hr		3 hr		5 hr		8 hr		27 hr	
Concentration	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C
400 µg/µL	ND	131.8*	ND	3.1	12.5	2.8	6.3	1.1	20.2	2.1	4.6	3.4
200 µg/µL	ND	294.6	ND	91.4	103.8	58.2	43.6	48.1	62.8	35.6	80.0	55.2
100 µg/µL	ND	727.7	ND	511.7	518.6	487.0	500.7	502.4	407.9	489.5	495.2	479.6
50 µg/µL	ND	859.8	ND	729.0	742.0	738.3	732.9	785.4	739.9	641.4	768.5	718.8

* DNA concentration (ng/µL) in the supernatant following centrifugation. ** ND: not done.

Table 8.3 Plasmid DNA concentration remaining in supernatant after plasmid DNA binding with alum/PBS diluent.

Time	0.5 hr		1 hr		3 hr		5 hr		8 hr		27 hr	
Concentration	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C
400 µg/µL	ND	1267.3	ND	1279.1	1283.6	1187.3	1191.1	1194.5	1179.4	1189.1	1201.2	1193.7
200 µg/µL	ND	1301.1	ND	1204.5	1190.1	1192.5	1199.1	1196.6	1200.9	1204.3	1214.4	1228.0
100 µg/µL	ND	1274.8	ND	1188.2	1176.7	1164.0	1175.5	1176.5	1173.6	1153.5	1179.5	1195.1

* DNA concentration (ng/µL) in the supernatant following centrifugation. ** ND: not done

Table 8.4 Effect of solvent on plasmid DNA concentration remaining in the supernatant after plasmid DNA binding with Phema adjuvant.

Solvent	100% dH_2O	50% alcohol/50% dH_2O	100% alcohol
DNA concentration (ng/µL)	505.9	208.8	28.3

8.3.2 Effect of adjuvants on antibody response

No HI titre was detected in any Hy-Line chickens 3 weeks post the second vaccination. By 10 days post virus challenge, all birds sero-converted with a range of HI titres as shown in Table 8.5. There was a statistically significant difference ($P<0.05$) in the GMT of HI antibody between pre- and postchallenge in all vaccinated groups and the naïve control group using the paired-sample T test.

There was no significant difference (ANOVA, $P>0.5$) in HI antibody GMT post-challenge between the naïve control group and any of the vaccinated groups, nor was there any significant difference (ANOVA, $P>0.5$) between the pCAG-HAk group and any of six pCAG-HAk adjuvanted groups.

Table 8.5 Antibody response prior to and post virus challenge.

	Naive control	pCAG-HAk								
		No adjuvant	Alum			Essai	Phema			
			NaCl	PBS	subtotal		200µg	100 µg	10 µg	subtotal
Prior	0	0	0	0	0	0	0	0	0	0
Post	6.0±2.0*	5.6±1.7	6.8±2.4	5.4±2.0	6.1±2.2	6.8+2.6	5.3±1.5	5.3±1.5	6.6±2.1	5.8±1.8

* Values represent GMT (log_2) ± SD of each group. The data in the naïve control and pCAG-HAk groups was taken from Chapter 7.

8.3.3 Effect of adjuvants on virus shedding

As shown in Table 8.6, in comparison with the naïve control group, the pCAG-HAk vaccinated group reduced the virus excretion rate from 70.8% to 45% in OS post challenge and from 12.5% to 0 in CS. However, the pCAG-HAk vaccinated group was not significantly different from the naïve control group in OS and CS. Possibly, the

pCAG-HAk vaccine may have elicited some borderline level of protective immunity in the oropharynx (Chi square, P= 0.083).

In comparison with the naïve control group, the alum-NaCl adjuvanted pCAG-HAk group showed a reduction in virus shedding rate from 70.8% to 40% in OS post challenge and showed no virus shedding in CS. There was a significant difference (Chi square, P= 0.04) for OS between the naïve control group and the alum-NaCl adjuvanted pCAG-HAk group. The alum-PBS adjuvanted pCAG-HAk group was also significantly (Chi square, P= 0.018) different from the naïve control group in frequency of virus shedding in OS. However, there was no significant difference in virus shedding in OS and CS between the pCAG-HAk vaccinated and either the alum-NaCl or alum-PBS adjuvanted groups. Neither was there a significant difference between the alum-NaCl and alum-PBS groups.

Although the Essai adjuvanted pCAG-HAk group showed less virus shedding in both OS and CS than the naïve control group, there was no significant difference between them. Neither was there a significant difference between the pCAG-HAk and the Essai adjuvanted groups.

There was a significant difference (Fisher's exact test, P=0.014) between the naïve control group and the 100μg Phema adjuvanted pCAG-HAk group in the rate of virus shedding in OS but no significant difference in CS. However, the 200μg or 10 μg Phema adjuvanted pCAG-HAk groups was not significantly different in virus shedding from the naïve control group. Furthermore, there was no significant difference in virus shedding between the pCAG-HAk group and the 200μg, 100 μg or 10 μg Phema adjuvanted groups.

Table 8.6 Level of virus shedding in chickens vaccinated with pCAG-HAk plasmid with various adjuvants following virus challenge*.

	Naïve control	pCAG-HAk						
		No adjuvant	Alum		Essai	Phema		
			NaCl	PBS		200μg	100 μg	10 μg
OS	17/24^{a}(70.8)	9/20^{ab}(45)	8/20^{bc}(40)	7/20^{bc}(35)	10/20^{ab}(50)	8/16^{ab}(50)	3/12^{bc}(25)	9/20^{ab}(45)
CS	3/24 (12.5)	0/20 (0)	0/20 (0)	3/20 (15)	2/20 (10)	1/16 (6.3)	0/12 (0)	1/20 (5)

* Number of swabs positive for virus isolation/total number of swabs tested. Percentage rate is shown in parentheses. OS and CS refer to oropharyngeal and cloacal swabs respectively.
The data in the naïve control and pCAG-HAk groups was taken from Chapter 7.
For the OS row, different lowercase superscript letters indicate statistical differences ($P < 0.05$) within the row using Chi square or Fisher's exact test. There were no significant differences between groups for the CS.

8.4 Discussion

Following the previous trial using lipofectin as the adjuvant, another attempt was made to compare different chemicals as DNA vaccine adjuvants, namely alum, Phema and Essai. Although no statistical differences in virus shedding in OS and CS occurred between the pCAG-HAk group and its corresponding adjuvanted groups, a significant reduction in virus shedding in the oropharynx was evident in both alum groups and the 100 μg Phema group compared with the naïve control group which was not shown by the non-adjuvanted pCAG-HAk group. This suggested that either alum or Phema as adjuvant further augmented the immune response induced by the pCAG-HAk construct. However, Essai did not show any apparent effect. In an H5 avian influenza DNA vaccine study on adjuvant comparisons, two different cationic liposomes (lipofectin and lipotaxi) improved antibody titres, whereas, the other chemical adjuvants (including 25% sucrose, diethylaminoethyl dextran, calcium phosphate, polybrene) decreased the antibody response (Suarez and Schultz-Cherry 2000a). These results also illustrated that chemical

adjuvants for DNA vaccines are not always effective. However, the effectiveness of Phema or other nanoparticles as new adjuvants warrants additional investigation.

Aluminum hydroxide has a net positive charge in 0.9% NaCl (pH 7.0) and hence alum-NaCl binds to the negatively charged plasmid DNA. Aluminum hydroxide in PBS possibly reacted to produce aluminum phosphate, which would have conferred a net negative charge to the surface of alum and prevented binding to DNA. This explains why alum-NaCl binds DNA and alum-PBS does not. Ulmer *et al.*(1999) reported that non-DNA-binding aluminum salts (DNA-aluminum phosphate formulations) significantly enhanced antibody titres in mice, guinea pigs and Rhesus monkeys compared with naked DNA. In contrast, DNA/aluminum hydroxide formulations (DNA binding aluminum salt) were less potent than naked DNA. Our results showed that both DNA-binding (alum-NaCl) and non-DNA-binding (alum-PBS) aluminum-based adjuvants enhanced immune responses initiated by the pCAG-HAk DNA vaccine in terms of frequency of virus shedding in the oropharynx, although there was no significant difference between these two groups. The difference in the effectiveness of the different alum adjuvants between these two experiments may be due to the differences in the indicators used to determine efficacy of DNA vaccines. In Ulmer's study antibody titre was used, whereas, virus shedding frequency was used in this study. Direct comparison of the effect of these adjuvants on antibody responses could not be carried out due to the problem encountered with the Hy-Line chickens which did not produce antibody responses to the pCAG-HAk vaccines, even though reduction in virus shedding was evident in vaccinated chickens.

Aluminum compounds are currently the most commonly used adjuvants for protein vaccines due to their good safety record and low cost. However, their exact mechanisms

of action are not yet fully understood. They may act via several mechanisms. Aluminum compounds precipitate protein antigens to form a “depot” at the site of injection, which allows for a slow release of antigen (Cox and Coulter 1997; Gupta 1998). This hypothesis was supported by a pharmacokinetic experiment using aluminum adjuvants in rabbits in which it took 28 days to dissolve about 50% of the aluminum phosphate at an IM injection site (Flarend *et al.* 1997). Presumably this may be the case for a DNA/alum-NaCl formulation but the depot effect would not apply for the non-binding DNA/alum-PBS adjuvant.

Aluminum salts/DNA formulations could protect plasmid DNA from degradation by nucleases *in vivo* and thus enhance immunogenicity. This protection was confirmed *in vitro* using aluminum phosphate (Ulmer *et al.* 1999), but this was challenged by the observations that other compounds, such as heparin or aurin tricarboxylic acid, did not enhance DNA vaccine immunogenicity *in vivo*, although they protected DNA *in vitro*.

Considering that aluminum phosphate/DNA formulations did not cause increased expression levels or longevity of antigen expression in muscle (Ulmer *et al.* 1999), the mechanism of action possibly involved targeting of aluminum-adsorbed DNA particles to APC due to the particle aluminum salt size (<10 μm) and its capacity to convert DNA immunogens to particulate forms.

Moreover, aluminum compounds also stimulate immune-competent cells possibly by activation of complement, induction of eosinophilia, release of inflammatory cytokines, and activation of macrophages. This may lead to a local inflammatory response and thus non-specifically activate the whole immune system (Gupta 1998).

The use of particulate carriers as novel vaccine delivery systems is an area currently receiving a high level of interest. Micro- and nano-size particle characteristics, such as size and surface properties, including surface charge and hydrophobicity, affect vaccine efficacy (Xiang *et al.* 2006). Recently, micro- and nano- size particles have been used as experimental adjuvants in animal models. Some studies have shown that nanoparticles might be more successful in stimulating immune responses *in vivo* than microparticles (Evans *et al.* 2004; O'Hagan *et al.* 2004; Singh *et al.* 2000). A range of inert nanoparticles have been tested and shown to be effective delivery vehicles for protein and peptide antigens. Nevertheless, application of nanoparticles with DNA vaccines is only at the exploratory stage (Cui and Mumper 2003a; Minigo *et al.* 2007). A biodegradable copolymer Phema has been used in a drug delivery system (Piotrowicz and Shoichet 2006; Rao 1998). However, there appears to be no previous reports using Phema for vaccine adjuvant purposes.

Due to the hydrophilicity of Phema, it is suspected that the ethanol solvent used in the current study may have imparted positive surface charges and allowed more absorption of the plasmid DNA than with water as a solvent. Plasmid DNA was possibly absorbed to the surface of Phema through electrostatic interaction or covalent binding and this did not appear to damage the DNA’s biological functions. The interaction also appeared to be complex because the optimum effect was achieved with the 100µg dose group and the response was poorer with both the 10 and 200µg dose groups. The mechanisms behind the observed adjuvant effect have not yet been resolved. Possible mechanisms are that these nanocarriers prevent DNA degradation and facilitate targeted delivery to APC (Khatri *et al.* 2008; Singh *et al.* 2000).

Effective adjuvants are able to facilitate uptake of antigen by DCs and to promote the activation of these cells *in vivo* (Scheerlinck *et al.* 2006). The size of nanoparticles used as DNA carriers has been shown to be of importance for DNA vaccine efficacy (Fifis *et al.* 2004a; Minigo *et al.* 2007; Scheerlinck *et al.* 2006). Conjugation of antigen to 40–50 nm nanoparticles elicited 2- to 10-fold higher $CD8^+$ T cell responses in mice than those induced by other bead sizes (Fifis *et al.* 2004a). One possible explanation was that particles of different sizes may target different APCs. This was verified in an experiment showing that the 40–50 nm particles targeted DCs, whereas, the smaller 20 nm particles localized preferentially in CD40+ cells and large particles (1.0 µm) predominantly localized in F4/80+CD80+ cells. Since DCs are the only APC capable of T cell priming (Banchereau and Steinman 1998), this size-dependent particle uptake might explain the higher efficacy of DNA/50 nm nanoparticles. In the current study, we did not measure the effect of Phema on cellular immune response, such as interferon production, although in other studies nanoparticles have potentiated cell mediated responses induced by DNA vaccines (Scheerlinck *et al.* 2006). Additionally, in this study the physicochemical characterization of Phema, such as particle size, surface charge, detergent content, release rate of DNA-nanoparticles and DNA integrity was not able to be conducted due to time and resource constraints. It was also clear that the DNA loading efficiency for Phema needs to be increased above the 30% estimated in this pilot study. Whether the use of 40 to 50 nm Phema nanoparticles (rather than the larger particles used in this study) would further enhance immune responses should be further investigated.

The pCAG-HAk vaccines with or without adjuvants did not induce a measurable antibody response in the Hy-Line chickens, however, the pCAG-HAk vaccines with alum

or Phema adjuvants gave some enhanced reduction in virus shedding from the oropharynx. The problem of lack of antibody response in Hy-Line chickens to DNA vaccines will require further investigation.

In the present study it has been shown for the first time that Phema has provided some evidence of an enhancing or adjuvant effect when used with a DNA vaccine in chickens. This study also appears to be the first to report that traditional aluminum hydroxide adjuvants, either binding or non-binding, show evidence of enhancement of immune responses in chickens, resulting in reduction of virus shedding in the oropharynx from chickens challenged with an H6N2 influenza A virus.

Chapter 9

General Discussion and Conclusions

Since the first DNA vaccines were described, they began to enjoy great scientific popularity with largely two focuses: applying DNA vaccination to various model systems and improvement of DNA vaccine efficacy (Bergmann-Leitner and Leitner 2004). Despite a number of approaches addressing parameters that limit immune responses to DNA vaccines, there is little data to indicate which of these approaches will be most useful and practical for use with commercial vaccines (Greenland and Letvin 2007).

This study focused on the development of a DNA vaccine for chickens and improvement of vaccine efficacy of the DNA vaccine based on AIV A/coot/WA/2727/79 (H6N2) as a proof-of-concept study for DNA vaccines against AI viruses. Initial biological and genetic characterization of this virus, which was to be used for virus challenge studies in vaccinated chickens, was conducted and described in Chapter 2. Different approaches to enhance the immunogenicity of the DNA vaccine in a chicken model were assessed and reported in Chapters 3 to 8. The study evaluated the effectiveness of different DNA vectors encoding HA or NP genes of the virus in chickens and demonstrated synergistic effects from various manipulations of the DNA vaccines. In particular, selection of an appropriate expression vector, the insertion of an enhancer sequence into a vector backbone, and the use of electroporation as the method of delivery were the most effective strategies. Use of chemical adjuvants such as lipofectin, alum and nanoparticles further enhanced plasmid-induced immune responses.

Specific findings from the study are discussed below. Although different approaches were used and discussed in each chapter, some issues need to be emphasized with a view to indicating future directions that could be followed with this research.

The ability of AI viruses to produce CPE in MDCK cells in the absence of trypsin correlates positively with pathogenicity (Shankar *et al.* 2009). Low pathogenic AI viruses cannot form plaques in the absence of trypsin, but the addition of trypsin to the cells will allow the cleavage of HA and produce plaquing (Bosch *et al.* 1981). In conducting the characterization of the LPAI A/coot/WA/2727/79 (H6N2) virus, it was observed that this virus was able to replicate moderately well in MDCK cells in the absence of exogenous trypsin, although the addition of trypsin did allow the virus to replicate to a higher titre in the cells. This adaption of a LPAI H6N2 virus from a wild bird to grow readily in MDCK cells was interesting and raised the question of how easily such a virus could change to replicate *in vivo* in mammalian species. It should be noted, however, that this adaption did not translate into a higher level of virulence for chickens challenged with the virus in this study.

Initially, the study evaluated five commercial plasmid vectors for the preparation of DNA construct vaccines encoding the HA of the virus and the immunogenicity of the resultant constructs were compared. The potential of a DNA vaccine construct to induce an immune response *in vivo* has been shown to correlate positively with the level of expression of the encoded protein *in vitro* (Jiang *et al.* 2007; Melkebeek *et al.* 2007; Steinberg *et al.* 2005). This is likely to be used as an indicator for selection of potential DNA vaccines before *in vivo* assessment, which is useful in terms of time-saving, cost reduction and animal welfare considerations. These studies supported this selection

process as the level of *in vitro* expression measured by IFT or WB did correlate with the level of *in vivo* response to the vaccines.

A variety of factors determine the magnitude and type of immune response induced by plasmid DNA vaccines. With analogy to conventional vaccines, the main factors affecting the efficacy of a DNA vaccines are the nature of a plasmid-expressed antigen, and the intrinsic adjuvant properties of the DNA expression vector (Bergmann-Leitner and Leitner 2004; Deml *et al.* 2001). In addition to these two major factors, other parameters, such as immunization methods and route, dose of plasmid DNA, number of vaccinations and vaccination intervals all play an important but less critical role in the context of the plasmid-induced immune response (Leitner *et al.* 1999). In the current studies involving the evaluation of different approaches to enhance immunity, selection of different expression vectors, together with incorporation of a Kozak sequence were shown to be important factors in increasing the expression of transgenes in DNA immunized animals. Codon optimization of target gene has been explored to improve the immunogenicity of DNA vaccines due to the codon bias of the hosts. Although codon optimization of the HA gene did not in itself enhance the immune response over the level achieved by the pCAGGS vector with a Kozak sequence, it may still be worth further investigation to improve the expression of DNA vaccines in some host sytems. However, this approach is difficult, time-consuming, and expensive.

Optimization of dose rates, vaccination times and intervals and combination with adjuvants have largely played a secondary role in successful vaccination. In this study, different doses for the five DNA constructs were assessed and although immune responses were generally poor for all vectors, it appeared that a dose of 100µg plasmid

DNA seemed to be the most effective in the chicken model. Increasing the number of vaccinations, in combination with an adjuvant, augmented plasmid-induced immune responses with the VR1012 vector as described in Chapter 3, but the effect on the level of immune response from multiple doses was variable and limited. Three-week vaccination intervals were used for the initial vaccinations reported in chapter 3 and in chapters 5, 6 and 8; and a 4-week vaccination interval was used for the layer chickens vaccinated with three alternate plasmid vectors reported in Chapter 4. No antibodies or a very low level of measurable antibody responses were detected in the Hy-Line layer chickens that were vaccinated in these studies, however, the SPF chickens vaccinated at a 3 week interval with the pCAG-HAk construct in Harbin gave moderate HA antibody responses. This variability of antibody response induced by the pCAG-HAk construct in the different chicken breeds may provide further insights into the efficacy of DNA vaccination and would have been followed up in this study if time and resources had permitted.

The DNA vaccine studies in the Hy-Line chickens were disappointing as far as induction, or enhancement of antibody responses was concerned. Booster doses of the DNA vaccine using the VR1012 construct administered with lipofectin adjuvant did result in some chickens producing antibody responses to HA, although the subsequent studies using two doses of the more effective pCAG-HAk construct with three other adjuvants in Hy-Line chickens did not induce antibody to the HA. However, the use of the adjuvants lipofectin, alum and Phema nanoparticles did enhance the effectiveness of the vaccines in reducing virus shedding from the oropharynx after challenge. It is apparent from these studies that there needs to be an antibody response to the DNA vaccine itself before one can expect significant enhancement via the use of adjuvants. Unfortunately for logistical and

resource reasons it was not possible during these studies to conduct adjuvant studies with the pCAG-HAk vaccine in SPF chickens.

DNA vaccines appear to act as their own adjuvant owing to the presence of ISS in their backbone (Davis 1997), thus DNA vaccines can be effective without adjuvants. However, incorporation of either chemical or genetic adjuvants to DNA vaccines does modulate or enhance their efficacy. In this study, lipofectin (liposome), aluminium hydroxide (traditional adjuvant), Essai (microparticle) and Phema (nanoparticle) were assessed. With the exception of Essai, all the chemicals demonstrated measurable adjuvant effects. Since lipofectin is too expensive to allow its routine use, the traditional aluminium hydroxide adjuvant, either binding or non-binding with DNA, may be useful as an adjuvant for enhancing DNA-induced immune responses in chickens owing to its low price and safety record. To our knowledge, nanoparticles have not been used for the development of AI DNA vaccines previously. In this study, a novel nanoparticle Phema used as an adjuvant showed promising results, comparable with alum adjuvants, for a DNA vaccine. The potential of nanoparticles as adjuvants has not been extensively explored with DNA vaccines and they are worthy of further investigation due to their biodegradability, biocompatibility, lack of side-effects and ease of delivery (Scheerlinck and Greenwood 2006). As DNA vaccination induced either Th1- or Th2-biased immune responses, use of nanomaterials of varying size, zeta potential and formulation could be developed and tested as alternative delivery systems to enhance or target the specific immune responses required (Fifis *et al.* 2004a; Minigo *et al.* 2007). Another alternative for future study is the use of genetic adjuvants whereby plasmid constructs expressing

different cytokines or co-stimulatory molecules can be combined with antigen specific DNA vaccines (Manoj *et al.* 2004; Scheerlinck 2001).

Direct IM injection is a very practical and convenient method for vaccine administration in the field (Chen *et al.* 2001). Compared to the transfection rate in mice, IM injection of DNA vaccines in larger animals or humans is lower and thereby, has resulted in lower efficacy of DNA vaccines, although the reasons for this are not currently understood (Babiuk *et al.* 2003). Electroporation was shown to substantially intensify delivery of DNA to cells, leading to increased expression and elevated immune responses (Widera *et al.* 2000). In Chapters 4 and 5, higher efficacy of HA-expressing DNA vaccination was observed via EP than via IM injection. Although increasing the level of gene expression by manipulation or optimization of the plasmid vector, or by increasing the number of transfected cells through improved delivery systems, may separately result in improved efficacy of DNA vaccines, these two approaches are complementary and synergistic and a combination of these two approaches may be the most effective for achieving optimal DNA immunization (Babiuk *et al.* 2003).

Although a large number of DNA vaccine studies have been conducted, many of the results are difficult to compare and are inconsistent (Leitner *et al.* 1999). Indeed, there is no universal protocol which applies to all DNA vaccines (Bergmann-Leitner and Leitner 2004). From the results of these and other studies, one can conclude that it is necessary to optimize every newly developed DNA vaccine for maximum effectiveness and this appears to be a clear limitation for DNA vaccines.

One attribute of DNA vaccines is that it is possible to manipulate and/or formulate the construct to induce immune responses that are most appropriate for specific pathogens

(Babiuk *et al.* 1999b). Although DNA immunization, in most cases, induces a predominately Th1 response (Babiuk *et al.* 1999b; Raz *et al.* 1996), mixed Thl and Th2 responses are recognised (Davis 1997). Whether a Thl or Th2 response predominates can be achieved by altering the method or route of DNA administration (Feltquate *et al.* 1997; Pertmer *et al.* 1996; Torres *et al.* 1997), altering the form of the antigen (secreted, cytoplasmic or membrane bound) (Lewis *et al.* 1996), the dose of antigen (Barry and Johnston 1997) or by co-administration of plasmids encoding various cytokines or co-stimulatory molecules (Lewis *et al.* 1997).

The experiments with five DNA construct vaccines encoding the H6 HA gene reported in this thesis showed that these vaccines gave low to undetectable levels of HA antibody but produced evidence of protective immunity in chickens challenged with the homologous LPAI virus, as demonstrated by reduction of virus shedding from the oropharynx and cloaca in challenged chickens. Similar results with poor antibody responses, but protection from challenge have been reported in other studies with influenza viruses in mice or chickens (Fynan *et al.* 1993a; Fynan *et al.* 1993b; Kodihalli *et al.* 1997; Robinson *et al.* 1993). Immune responses to antigens expressed by the plasmid DNA transfected host cells are mediated by the synergistic activity of B cells and T cells. Antibody responses to HA protein are critical for virus neutralization and prevention of influenza virus infection (Brown *et al.* 1992; Webster *et al.* 1991), whereas, CTL responses play a role in the clearance of influenza virus infection (McMichael *et al.* 1983). Intramuscular immunization of mice with an HA-expressing plasmid demonstrated that neutralizing antibodies and not CTLs mediated protection against lethal virus challenge (Operschall *et al.* 2000). In murine studies it is hypothesized that DNA immunization induces a

memory response, and protection appears to be mediated by the priming of both T-helper and B-cell memories, resulting in a secondary immune response in response to the challenge (Fynan *et al.* 1995). Both cellular and humoral responses contribute to protection against influenza virus infection (Robinson *et al.* 1997). The low immunogenicity of DNA vaccines, demonstrated by the presence of low antibody levels, is not always an indicator of protective immunity (Scheerlinck 2001). In fact, DNA vaccines were particularly effective in priming anamnestic immune responses and the B-cell memory response was often sufficient to protect animals from challenge (Babiuk *et al.* 1999b; Fynan *et al.* 1993a; Kodihalli *et al.* 1999; Kodihalli *et al.* 1997; Kodihalli *et al.* 2000; Robinson *et al.* 1993). This characteristic of DNA vaccines makes challenge of vaccinated animals with the relevant pathogen imperative when evaluating these vaccines (Scheerlinck 2001). Indeed, in the majority of studies of DNA immunization so far, animals were challenged to measure the level of plasmid-induced protection generated by DNA vaccination (Babiuk *et al.* 1999b). For logistical reasons it was not possible to conduct studies of CMI responses to the DNA vaccines post-challenge in the chickens in this study, however with the very limited HA antibody responses to the DNA vaccines with four of the five vectors, yet evidence of protection from challenge demonstrated by reduced virus shedding, one can speculate that the vaccines had stimulated cellular immune responses to a certain degree.

The pCAG-HAk construct generated measurable HI titres in SPF chickens (Chapter 5). Nonetheless, in all experiments using Hy-Line chickens, two immunizations with either VR-HA, pCI-HA, pCI-neo-HA, pCI-HAk or pCAG-HAk plasmids showed no antibody response or the production of a very low level of HI antibody. This important difference

in chicken strain effect was discussed in detail in Chapter 7 and would have been the focus for further investigation if time and resources had permitted. However, with the most efficient pCAG-HAk construct evaluated in SPF chickens, there was only 75% - 87.5% seroconversion rate following two immunizations (Chapter 5). This highlighted that DNA vaccines containing the basic plasmid construct only induced low or moderate antibody responses in chickens and that other factors such as the delivery route and adjuvants may be necessary to produce strong antibody responses. Other DNA vaccine studies such as AI in chickens (Fynan *et al.* 1993b; Kodihalli *et al.* 2000; Robinson *et al.* 1993), canine parvovirus in dogs (Babiuk *et al.* 1999b), haematopoietic necrosis virus in fish (Corbeil *et al.* 2000) and influenza in mice (Fynan *et al.* 1993b; Johnson *et al.* 2000; Kodihalli *et al.* 1999) are consistent with this conclusion.

The prevention of clinical disease and death in vaccinated chickens challenged by various HPAI viruses is well documented (Swayne 2009). However, few studies have addressed LPAI challenge. It was reported previously that some waterfowl-origin influenza virus strains were nephrotropic and enterotropic following intravenous challenge (Slemons and Swayne 1990, 1995). However, no virus was recovered from kidneys in naïve chickens via oral and oculonasal challenge with the H6N2 virus in the current studies (Chapter 2). In some studies with LPAI vaccines, in order to increase LPAI virus multiplication and shedding in chickens after challenge, co-infection with *Mycoplasma* spp. and *Salmonella* spp., as well as high doses of oculonasally inoculated LPAI virus have been used to assess the vaccine potency (Cherbonnel *et al.* 2003; Le Gall-Recule *et al.* 2007; Rousset *et al.* 2003). In another study, vaccinated ducks were only subjected to high dose of LPAI virus challenge by nasal and intraocular instillation (Prel *et al.* 2007). As these proof-of-

concept studies were conducted using a H6N2 LPAI virus which was shown in initial characterization studies to cause no clinical or pathological changes, it was decided to use level of virus shedding after challenge with homologous virus as an indicator of protection in the current studies.

The frequency and titre of virus shedding after challenge is consistently used as an indicator to assess the potency and efficacy of viral vaccines. With vaccines against HPAI viruses, challenge studies use both protection rate and measurement of virus shedding after HPAI virus challenge to evaluate the potency of vaccines, but with LPAI challenge, virus shedding gives an option to measure protection. In a number of HPAI challenge studies in chickens, the post challenge shedding is reported on day 3 (Kodihalli *et al.* 1997; Swayne *et al.* 2000b) or day 7 post-challenge (Chen *et al.* 2001) whereas in these challenge studies with the H6N2 LPAI virus, shedding was assessed by collection of cloacal and oropharyngeal swabs on a daily or alternate day basis. Virus shedding through the cloaca and oropharynx is a major source of AI virus contamination of the environment (Condobery and Slemons 1992; Crawford *et al.* 1998; Wood *et al.* 1994). Effective vaccine strategies to control AI require generation of specific immunity to preclude or clear virus replication in the respiratory and gastrointestinal tracts at the mucosal level and hence prevent virus shedding and bird-to-bird horizontal transmission (Swayne *et al.* 1997).

In this study, a total of three virus challenge experiments for assessment of the efficacy of DNA vaccines were performed through simultaneous intraocular, intranasal and oral inoculation. The challenge doses in the experiments described in Chapters 3, 4 and 7 were $10^{7.25}$ (0.5 mL), $10^{6.25}$ (1 mL), and $10^{6.5}$ (0.5 mL) EID_{50} /0.1 mL, respectively. Our

three separate challenge protocols produced comparable results. Either the naïve or empty vector vaccinated control birds intermittently shed virus following H6N2 virus infection. The virus shedding for unvaccinated controls gave similar results across the three experiments with the frequency of virus recovery from the oropharynx (58.7% - 54 chickens positive for virus shedding out of a total of 92 chickens tested) higher than that from the cloacal swabs (27.2% - 25/92). In comparison with occasional virus shedding in either oropharyngeal or cloacal swabs in the inactivated vaccine control group, the naïve control or empty vector control groups displayed higher frequencies of virus shedding. However, in the LPAI challenge study in Harbin that used a challenge dose of 0.2 mL ($EID_{50}10^{6.17}$) of A/duck/Anhui/269/2002 (H6N2) by intranasal instillation, three out of six SPF chickens intermittently excreted virus in oropharyngeal swabs only in the first four days, whereas, no virus shedding was present in cloacal swabs during the seven consecutive days over which swabs were collected. This suggested that high dose inoculation of LPAI via multiple routes into chickens was necessary to induce cloacal virus shedding.

In live recombinant vector-based vaccine studies with HPAI virus challenge, survival of chickens did not coincide with complete lack of virus shedding from the oropharynx or cloaca (Crawford *et al.* 1999; Swayne *et al.* 2000a). In an HA-expressing DNA vaccine study, GG immunization of chickens with DNA vaccines afforded high levels of protection against homologous H5 virus and its antigenic variants with the absence of virus shedding in either the trachea or cloaca at day 3 after virus challenge. However, whether vaccinated birds occasionally shed virus on other days was unknown (Kodihalli *et al.* 1997). With vaccination against H5N1 HPAI viruses, protection of chickens from

disease correlated with approximately 1000-fold reduction in the titre of virus shed from the oropharynx compared with unvaccinated controls on day 2 post-challenge (Ellis *et al.* 2006). In a murine study with plasmid DNA encoding influenza viral proteins, virus titre in the lung of surviving mice after a lethal A/PR/8/34 (HlNl) virus challenge still reached $10^{3.1}$ EID_{50}/mL (Chen *et al.* 1998b). The statistically significant difference in virus titre in lungs between the vaccinated and control groups directly correlated with the survival of mice subjected to a lethal A/PR/8/34 (HlNl) virus challenge. In other words, the vaccinated group was regarded as protected even though surviving mice had low levels of virus in lung tissue for a period after challenge. In the context of LPAI challenge therefore, it is reasonable to presume that a significant reduction of virus shedding in vaccinated birds compared to unvaccinated controls is consistent with a degree of protection.

Comparison of the level of virus shedding between groups was made on the basis of frequency of positive swabs over the whole post-challenge period rather than comparison of virus titres because the titres of virus shed even in unvaccinated controls were very low. As compared with the control group, the groups receiving the VR-NP and VR-HA constructs as outlined in Chapter 3, and the pCAG-HAk construct outlined in Chapter 8, showed significant reduction in virus shedding in oropharyngeal swabs while the pCI-HA, pCI-neo-HA and pVAX-HA constructs described in Chapter 4 showed significant reduction in virus shedding in cloacal swabs. Therefore, although all the DNA construct vaccines gave some reduction in virus shedding following challenge, the extent of reduction in virus shedding was inconsistent. However, the adjuvanted 100 μg VR-HA group showed a similar reduction in virus shedding to the inactivated virus vaccine group

(55.6% reduction for cloacal swabs and 66.7% reduction for oropharyngeal swabs), which also gave a significant anti-H6 HA antibody response pre-challenge. Moreover, the adjuvanted VR-NP group, the 100 µg naked or adjuvanted 100 µg pCI-HA group, the naked 100 µg pCI-neo-HA group, the 50 µg pVAX-HA, as well as the naked 100 µg pCAG-HAk, and alum-NaCl adjuvanted or phema adjuvanted pCAG-HAk groups showed no virus shedding in cloacal swabs, as was the case for the inactivated vaccine. Therefore, it appears possible that immunization with an effective HA-expressing DNA vaccine could provide equivalent protection to an inactivated virus vaccine and this would potentially reduce or prevent transmission of AIV under field conditions.

Another strategy that has been used with DNA vaccines is DNA vaccine priming followed by boosting with a protein-based vaccine (prime-boost strategy) and this has proven to be successful in improving the efficacy of DNA vaccines (Huber *et al.* 2009; Lo *et al.* 2008; McConkey *et al.* 2003; McShane 2002; Sedegah *et al.* 2003; Sedegah *et al.* 1998). Evaluation of a prime-boost strategy to enhance immune responses to the H6 HA DNA vaccine was planned to be undertaken in the study trip to the Avian Influenza Reference Laboratory in Harbin, China. The HA open reading frame (ORF) from the A/coot/WA/2727/79 (H6N2) was inserted into the intergenic region between the P and M genes of the LaSota Newcastle Disease Virus (NDV) vaccine strain using procedures described previously (Ge *et al.* 2007) and recombinant NDV expressing the H6 influenza virus HA was successfully rescued using reverse genetics. This live recombinant vaccine was ready to provide HA antigen boosting as part of a prime-boost strategy with the H6 HA DNA vaccine, but due to time and logistical difficulties this could not be completed as part of this thesis.

In conclusion, as a proof-of concept study, DNA immunization with various H6 HA gene constructs was shown to be variably effective in inducing immune responses in chickens and with one construct, pCAG-HAk, in inducing moderate levels of antibody to H6 HA in SPF chickens. Different strategies had to be explored before the development of a DNA vaccine capable of generating significant levels of H6 HA antibody was achieved. The factors investigated to enhance immune responses included the type of plasmid vector, the target gene, modifications of the gene insert, the dose of plasmid DNA used, optimization of the immunization protocols, evaluation of different delivery methods and the use of adjuvants. Some synergistic effects that improved the efficacy of the H6 HA DNA vaccines were demonstrated. Nonetheless, low and variable antibody responses, variable levels of protection and considerable inter-individual variability in responses were evident. DNA vaccination does provide a new and valuable approach to the development of AI vaccines, and offers advantages in safety, speed, simplicity, and the ability to target both cellular and humoral immune responses with a broader reactivity than the currently used inactivated whole virus vaccines or recombinant vectored vaccines. If the disappointing potency of DNA vaccines can be improved and safety can be established, this technology platform could become an important tool in combating influenza, other infectious diseases and cancers in animals and humans.

Appendix 2.1 Sequencing results of avian influenza A/coot/WA/2727/79 (H6N2) virus.

HA gene (1723 bp)

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   1 CAAAAGCAGGGGAAAATGATTGCAATCATAATATTCGCGATAGTGGCCTCTACCAGCAAATCAGATAAGATCTGC
   1          10        20        30        40        50        60        70
  76 ATTGGATACCATGCCAACAACTCGACAACGCAAGTGGACACAATATTAGAAAAGAATGTGACAGTGACGCATTCA
  76     80        90        100       110       120       130       140
 151 ATTGAGCTCCTAGAGAGTCAAAAGGAGGAGAGATTATGCAGAGTATTAAATAAAGCCCCTTTGGATCTAAAGGGT
 151          160       170       180       190       200       210       220
 226 TGCACTATTGAAGGTTGGATTCTTGGAAATCCCCAATGTGACATCTTGCTTGGAGACCAAATTTGGTCTTACATA
 226     230       240       250       260       270       280       290
 301 GTGGAGAGACCTGGAGCCCAACATGGGATATGCTACCCAGGGGCATTAAATGAATTGGAAGAGTTGAAAGCACTC
 301          310       320       330       340       350       360       370
 376 ATTGGATCTGGAGAAAGAGTACAGAGATTTGAAATGTTTCCCAAAAGCACATGGACCGGAGTGGACACTGGCAGT
 376     380       390       400       410       420       430       440
 451 GGAGTTACGAAAGCCTGTTCCTACAACAGTGGCTCATCTTTCTACAGAAACCTTTTGTGGATAATAAAAACAAAA
 451          460       470       480       490       500       510       520
 526 TCTGCTGCATATCCAGTGATTAGGGGAACATACAATAATACTGGCTCCCAATCAATTCTATATTTCTGGGGTGTA
 526     530       540       550       560       570       580       590
 601 CACCATCCTCCTGATACCAATGAGCAAAATACTCTGTATGGTTCTGGTGATAGATATGTTAGAATGGGTACTGAG
 601          610       620       630       640       650       660       670
 676 AGCATGAACTTTGCCAAAAGTCCAGAAATAGCAGCGAGACCAGCTGTAAATGGGCAAAGAGGGAGGATTGATTAC
 676     680       690       700       710       720       730       740
 751 TATTGGTCTATATTGAAGCCAGGAGAAACCTTAAATGTAGAATCCAATGGCAACTTGATAGCTCCTTGGTATGCT
 751          760       770       780       790       800       810       820
 826 TACAAATTCATCAGTTCCAATAACAAGGGAGCTGTCTTCAAATCAAACCTTCCAATTGAAGATTGTGACACTGCC
 826     830       840       850       860       870       880       890
 901 TGTCAGACAGTAGCTGGAGCACTAAGGACAAACAAAACTTTCCAAAATGTTAGCCCTCTCTGGATTGGAGAATGT
 901          910       920       930       940       950       960       970
 976 CCCAAGTATGTTAAAAGTGATAGCCTAAGACTGGCAACTGGTCTGAGAAATGTCCCACAGGCAGAGACGAGAGGA
 976     980       990       1000      1010      1020      1030      1040
1051 TTGTTCGGGGCCATAGCTGGGTTTATAGAAGGAGGATGGACAGGCATGATAGATGGATGGTATGGCTACCACCAC
1051          1060      1070      1080      1090      1100      1110      1120
1126 GAGAACTCACAGGGATCAGGCTATGCAGCAGACAAAGAAAGTACCCAGAAAGCAATTGACGGGATCACCAATAAA
1126     1130      1140      1150      1160      1170      1180      1190
1201 GTCAATTCCATTATTGACAAAATGAACACACAATTTGAGGCAGTAGAGCATGAGTTCTCAAATCTAGAAAGAAGA
1201          1210      1220      1230      1240      1250      1260      1270
1276 ATAGACAACCTAAACAAGAGAATGGAAGATGGATTTCTAGATGTGTGGACCTACAATGCTGAACTTTTAGTTCTA
1276     1280      1290      1300      1310      1320      1330      1340
1351 CTGGAAAATGAGAGAACCTTGGACCTGCATGATGCCAATGTGAAGAACCTATATGAAAAAGTGAAATCACAATTG
1351          1360      1370      1380      1390      1400      1410      1420
1426 AGAGACAATGCAAAGGATTTGGGTAATGGGTGTTTTGAATTTTGGCATAAATGTGATGATGAATGCATCAACTCA
1426     1430      1440      1450      1460      1470      1480      1490
1501 GTTAAGAATGGCACGTATGACTACCCAAAATACCAAGACGAAAGCAAACTTAATAGGCAGGAAATAGATTCAGTG
1501          1510      1520      1530      1540      1550      1560      1570
1576 AAACTGGAAAATCTGGGTGTATATCAAATCCTTGCTATTTATAGTACGGTATCGAGCAGTCTAGTTTTGGTGGGA
1576     1580      1590      1600      1610      1620      1630      1640
1651 CTGGTCATTGCCATGGGTCTTTGGATGTGCTCGAATGGTTCAATGCAATGCAGGATATGTATATAATTAGGAA
1651          1660      1670      1680      1690      1700      1710      1720
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NP gene (1565 bp)

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   1 AGCAAAAGCAGGGTAGATAATCACTCACCGAGTGACATCCACATCATGGCGTCTCAAGGCACCAAACGATCTTAT
   1          10        20        30        40        50        60        70
  76 GAACAGATGGAAACTGGTGGAGAACGCCAGAATGCTACTGAGATCAGAGCTTCCGTTGGAAGAATGGTTGGTGGA
  76     80        90        100       110       120       130       140
 151 ATTGGAAGATTCTACATACAGATGTGCACTGAACTCAAGCTCAGTGACTATGAAGGAAGGCTGATCCAAAATAGC
 151          160       170       180       190       200       210       220
 226 ATAACAATAGAGAGAATGGTCCTTTCAGCATTTGATGAAAGGAGGAACAAATACCTGGAGGAGCATCCCAGTGCT
 226     230       240       250       260       270       280       290
 301 GGGAAAGATCCTAAGAAGACTGGAGGTCCAATCTACAGAAGGAGAGATGGAAAATGGATTAGAGAACTGATCCTA
 301          310       320       330       340       350       360       370
 376 TACGACAAAGAGGAGATCAGGAGAATCTGGCGCCAAGCGAATAATGGGGAGGATGCAACTGCTGGCCTTACCCAT
 376     380       390       400       410       420       430       440
 451 TTGATGATATGGCATTCCAACCTCAATGATGCCACTTACCAGAGGACAAGAGCCCTTGTGCGTACTGGGATGGAC
 451          460       470       480       490       500       510       520
 526 CCTAGAATGTGCTCTCTGATGCAAGGCTCAACTCTCCCAAGAAGGTCTGGAGCTGCTGGCGCTGCAGTAAAAGGA
 526     530       540       550       560       570       580       590
 601 GTCGGAACAATGGTGATGGAACTGATTCGGATGATAAAACGGGGAATCAATGATCGAAATTTCTGGAGAGGCGAA
 601          610       620       630       640       650       660       670
 676 AATGGAAGAAGAACAAGGATTGCCTATGAGAGAATGTGCAACATTCTCAAAGGGAAATTCCAAACAGCAGCACAA
 676     680       690       700       710       720       730       740
 751 AGAGCAATGATGGACCAGGTGCGAGAAAGCCGGAATCCCGGGAATGCTGAAATTGAAGATCTCATATTTCTGGCG
 751          760       770       780       790       800       810       820
 826 CGATCTGCACTCATCCTGAGAGGGTCAGTGGCCCACAAGTCCTGCCTACCTGCTTGTGTGTATGGACTGGCTGTA
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 826      830       840       850       860       870       880       890
 901 GCCAGTGGATATGACTTTGAGAGAGAAGGATACTCCCTAGTTGGAATAGATCCTTTCCGCCTACTCCAGAACAGC
 901           910       920       930       940       950       960       970
 976 CAAGTATTCAGCCTCATCAGGCCCAACGAAAATCCAGCACACAAGAGTCAATTGGTCTGGATGGCATGCCACTCT
 976      980       990      1000      1010      1020      1030      1040
1051 GCAGCATTTGAAGACCTGAGGGTGTCAAGCTTCATCAGAGGGACAAGGGTGGTCCCAAGAGGACAACTGTCCACC
1051          1060      1070      1080      1090      1100      1110      1120
1126 AGAGGAGTCCAAATCGCATCAAATGAGAACATGGAAACAATGGACTCTAGCACTCTTGAACTGAGAAGCAGGTAC
1126     1130      1140      1150      1160      1170      1180      1190
1201 TGGGCTATAAGGACCAGAAGCGGAGGGAACACAAATCAGCAGCGGGCATCAGCTGGACAAATCAGCGTACAACCC
1201          1210      1220      1230      1240      1250      1260      1270
1276 ACTTTCTCTGTGCAGAGGAGTCTCCCATTCGAGAGAGCAACCATTATGGCAGCATTTACCGGGAACACTGAAGGC
1276     1280      1290      1300      1310      1320      1330      1340
1351 AGAACATCTGACATGAGAACTGAGATCATAAGAATGATGGAGAGTGCCAGACCAGAAGACGTGTCCTTCCAAGGG
1351          1360      1370      1380      1390      1400      1410      1420
1426 CGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCTTCCTTTGACATGAGTAACGAGGGA
1426     1430      1440      1450      1460      1470      1480      1490
1501 TCTTATTTCTTCGGAGACAATGCAGAGGAGTATGACAATTAAAGAAAAATACCCTTGTTTCTACT
1501          1510      1520      1530      1540      1550      1560
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NA gene (1468 bp)

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   1 AGCAAAAGCAGGAGTTGAAAATGAATCCAAATCAGAAGACAACAACAATTGGCTCTGTCTCTCCAACCATCGCAA
   1           10        20        30        40        50        60        70
  76 CAACATGCTTTCTCATGCAGATTGCTATCCTAGTAACAACTGTGACACTACACTTCAAACAAAGTGAATGCAAAA
  76      80        90       100       110       120       130       140
 151 TCCCCTCGAACAATCAAGTAGTTCCATGTGAGCCGATCATAATAGAAAGGAACATAACCGAGATAGTGTATTTAA
 151           160       170       180       190       200       210       220
 226 ACAATACTACCATAGAAAAAGAAATTTGTCCAAAAGTAGTAGAATACAGGAATTGGTCAAAACCGCAATGTCAAA
 226      230       240       250       260       270       280       290
 301 TTACAGGGTTTGCTCCTTTCTCCAAGGACAACTCAATTCGGCTTTCTGCTGGTGGGGACATTTGGGTAACAAGAG
 301           310       320       330       340       350       360       370
 376 AACCTTATGTGTCATGCAGCCCCAATAAATGTTATCAATTTGCGCTTGGGCAGGGGACCACACTGGACAACAAAC
 376      380       390       400       410       420       430       440
 451 ACTCAAATGGCACAATACATGATAGAATCCCTCATAGAACCCTTTTAATGAACGAATTGGGTGTTCCGTTTCACT
 451           460       470       480       490       500       510       520
 526 TGGGAACAAAGCAAGTGTGCATAGCGTGGTCCAGCTCGAGCTGTCATGATGGGAAAGCATGGCTACATATTTGTG
 526      530       540       550       560       570       580       590
 601 TCACTGGGGATGATAGGAATGCAACTGCTAGTTTCATTTATGACGGGATGCTTGTTGATAGTATTGGTTCATGGT
 601           610       620       630       640       650       660       670
 676 CTCAAAATATTCTCAGAACTCAAGAGTCAGAATGTGTTTGCATTAATGGGACTTGTACTGTAGTAATGACTGATG
 676      680       690       700       710       720       730       740
 751 GAAGTGCTTCAGGAGTGGCTGACACTAGGATACTATTCATTAGAGAAGGGAAGATCGTTCATATCAGCCCATTAT
 751           760       770       780       790       800       810       820
 826 CAGGAAGTGCTCAGCATATAGAAGAATGTTCTTGTTATCCCCGATATCCAGACGTCAGATGTGTTTGCAGAGACA
 826      830       840       850       860       870       880       890
 901 ACTGGAAAGGTTCAAATAGGCCCGTTATAAATATAAATATGGCAGATTATAGCATTGATTCCAGTTACGTATGCT
 901           910       920       930       940       950       960       970
 976 CAGGACTTGTCGGCGACACACCAAGGAACGATGATAGCTCTAGCAGCAGCAACTGCAGAGATCCTAATAATGAAA
 976      980       990      1000      1010      1020      1030      1040
1051 GAGGGAACCCAGGAGTGAAAGGATGGGCCTTTGACAATGGAAATGATGTTTGGATGGGTAGAACAATCAGCAAAG
1051          1060      1070      1080      1090      1100      1110      1120
1126 ATTCGCGCTCAGGTTATGAGACATTCAGAGTTATTGGTGGTTGGGCCACAGCTAATGCCAAGTCACAGATCAATA
1126     1130      1140      1150      1160      1170      1180      1190
1201 GGCAGATCATAGTTGACAATAATAACTGGTCTGGTTACTCTGGTATTTTCTCTGTTGAAGGCAAAAGCTGTATCA
1201          1210      1220      1230      1240      1250      1260      1270
1276 ATAGGTGTTTTTATGTGGAGTTGATAAGAGGGAGGCCGCAGGAAACTAGAGTATGGTGGACCTCAAACAGTATTG
1276     1280      1290      1300      1310      1320      1330      1340
1351 TCGTATTTTGCGGCACTTCAGGTACTTATGGAACAGGCTCATGGCCTGATGGGGCAAATATCAATTTCATGCCTA
1351          1360      1370      1380      1390      1400      1410      1420
1426 TATAAGCTTTCGCAATTTTAGAAAAAAACTCCTTGTTTCTACT
1426     1430      1440      1450      1460
```

*Underlined areas refer to extra sequence outside that listed in GenBank. Underlined bold sequence refers to cleavage site in HA.

Appendix 6.1 Comparison of HA and optiHA sequences.

```
HA              ATGATTGCAATCATAATATTCGCGATAGTGGCCTCTACCAGCAAATCAGATAAGATCTGC 60
OptiHA          ATGATCGCCATCATCATCTTCGCCATCGTGGCCAGCACCAGCAAGAGCGATAAGATCTGC 60
                ***** ** ***** ** ***** ** ******   ********    ************

HA              ATTGGATACCATGCCAACAACTCGACAACGCAAGTGGACACAATATTAGAAAAGAATGTG 120
OptiHA          ATCGGCTACCACGCCAACAACAGCACCACCCAGGTGGATACCATCCTGGAGAAGAACGTG 120
                ** ** ***** *********    ** ** ** ***** ** **  * ** ***** ***

HA              ACAGTGACGCATTCAATTGAGCTCCTAGAGAGTCAAAAGGAGGAGAGATTATGCAGAGTA 180
OptiHA          ACCGTGACCCACAGCATCGAGCTGCTGGAGAGCCAGAAGGAGGAGAGACTGTGCAGAGTG 180
                ** ***** **     ** ***** ** ***** ** ************ * ********

HA              TTAAATAAAGCCCCTTTGGATCTAAAGGGTTGCACTATTGAAGGTTGGATTCTTGGAAAT 240
OptiHA          CTGAACAAGGCCCCCCTGGATCTGAAGGGCTGCACCATCGAGGGCTGGATCCTGGGCAAC 240
                 * ** ** *****  ******* ***** ***** ** ** ** ***** ** ** **

HA              CCCCAATGTGACATCTTGCTTGGAGACCAAATTTGGTCTTACATAGTGGAGAGACCTGGA 300
OptiHA          CCCCAGTGCGATATCCTGCTGGGCGATCAGATCTGGAGCTACATCGTGGAGAGACCCGGC 300
                ***** ** ** *** **** ** ** ** ** ***   ***** *********** **

HA              GCCCAACATGGGATATGCTACCCAGGGGCATTAAATGAATTGGAAGAGTTGAAAGCACTC 360
OptiHA          GCCCAGCACGGCATCTGCTACCCCGGCGCCCTGAACGAGCTGGAGGAGCTGAAGGCCCTG 360
                ***** ** ** ** ******** ** **  * ** **  **** *** **** ** **

HA              ATTGGATCTGGAGAAAGAGTACAGAGATTTGAAATGTTTCCCAAAAGCACATGGACCGGA 420
OptiHA          ATCGGCAGCGGCGAGAGAGTGCAGAGATTCGAGATGTTCCCCAAGAGCACCTGGACCGGC 420
                ** **    ** ** ***** ******** ** ***** ***** ***** ********

HA              GTGGACACTGGCAGTGGAGTTACGAAAGCCTGTTCCTACAACAGTGGCTCATCTTTCTAC 480
OptiHA          GTGGATACCGGCAGCGGCGTGACCAAGGCCTGCAGCTACAACAGCGGCAGCAGCTTCTAC 480
                ***** ** ***** ** ** ** ** *****   ********* ***       ******

HA              AGAAACCTTTTGTGGATAATAAAAACAAAATCTGCTGCATATCCAGTGATTAGGGGAACA 540
OptiHA          AGAAACCTGCTGTGGATCATCAAGACCAAGAGCGCCGCCTACCCCGTGATCAGAGGCACC 540
                ********  ******* ** ** ** **     ** ** ** ** ***** ** ** **

HA              TACAATAATACTGGCTCCCAATCAATTCTATATTTCTGGGGTGTACACCATCCTCCTGAT 600
OptiHA          TACAACAACACCGGCAGCCAGAGCATCCTGTACTTCTGGGGCGTGCACCACCCCCCCGAT 600
                ***** ** ** ***   ***     ** ** ** ******** ** ***** ** ** ***

HA              ACCAATGAGCAAAATACTCTGTATGGTTCTGGTGATAGATATGTTAGAATGGGTACTGAG 660
OptiHA          ACCAACGAGCAGAACACCCTGTACGGCAGCGGCGATAGATACGTGAGAATGGGCACCGAG 660
                ***** ***** ** ** ***** **     ** ******** ** ******** ** ***

HA              AGCATGAACTTTGCCAAAAGTCCAGAAATAGCAGCGAGACCAGCTGTAAATGGGCAAAGA 720
OptiHA          AGCATGAACTTCGCCAAGAGCCCCGAGATCGCCGCCAGACCCGCCGTGAACGGCCAGAGA 720
                *********** ***** ** ** ** ** ** ** ***** ** ** ** ** ** ***

HA              GGGAGGATTGATTACTATTGGTCTATATTGAAGCCAGGAGAAACCTTAAATGTAGAATCC 780
OptiHA          GGCAGAATCGATTACTACTGGAGCATCCTGAAGCCCGGCGAGACCCTGAACGTGGAGAGC 780
                ** ** ** ******** ***    **  ******* ** ** *** * ** ** **    *

HA              AATGGCAACTTGATAGCTCCTTGGTATGCTTACAAATTCATCAGTTCCAATAACAAGGGA 840
OptiHA          AACGGCAACCTGATCGCCCCCTGGTACGCCTACAAGTTCATCAGCAGCAACAACAAGGGC 840
                ** ****** **** ** ** ***** ** ***** ********    *** ********

HA              GCTGTCTTCAAATCAAACCTTCCAATTGAAGATTGTGACACTGCCTGTCAGACAGTAGCT 900
OptiHA          GCCGTGTTCAAGAGCAACCTGCCCATCGAGGATTGCGATACCGCCTGCCAGACCGTGGCC 900
                ** ** *****     ***** ** ** ** ***** ** ** ***** ***** ** **

HA              GGAGCACTAAGGACAAACAAAACTTTCCAAAATGTTAGCCCTCTCTGGATTGGAGAATGT 960
OptiHA          GGCGCCCTGAGAACCAACAAGACCTTCCAGAACGTGAGCCCCCTGTGGATCGGCGAGTGC 960
                ** ** ** ** ** ***** ** ***** ** ** ***** ** ***** ** ** **

HA              CCCAAGTATGTTAAAAGTGATAGCCTAAGACTGGCAACTGGTCTGAGAAATGTCCCACAG 1020
OptiHA          CCCAAGTACGTGAAGAGCGATAGCCTGAGACTGGCCACCGGCCTGAGAAACGTGCCCCAG 1020
                ******** ** ** ** ******** ******** ** ** ******** ** ** ***

HA              GCAGAGACGAGAGGATTGTTCGGGGCCATAGCTGGGTTTATAGAAGGAGGATGGACAGGC 1080
OptiHA          GCCGAGACCAGAGGCCTGTTCGGCGCCATCGCCGGCTTCATCGAGGGCGGCTGGACCGGC 1080
                ** ***** *****  ******* ***** ** ** ** ** ** ** ** ***** ***

HA              ATGATAGATGGATGGTATGGCTACCACCACGAGAACTCACAGGGATCAGGCTATGCAGCA 1140
OptiHA          ATGATCGATGGCTGGTACGGCTACCACCACGAGAACAGCCAGGGCAGCGGCTACGCCGCC 1140
                ***** ***** ***** *****************   *****     ***** ** **

HA              GACAAAGAAAGTACCCAGAAAGCAATTGACGGGATCACCAATAAAGTCAATTCCATTATT 1200
```

```
OptiHA          GATAAGGAGAGCACCCAGAAGGCCATCGATGGCATCACCAACAAGGTGAACAGCATCATC 1200
                ** ** ** ** ******** ** ** ** ** ******** ** ** **   *** ** 

HA              GACAAAATGAACACACAATTTGAGGCAGTAGAGCATGAGTTCTCAAATCTAGAAAGAAGA 1260
OptiHA          GATAAGATGAACACCCAGTTCGAGGCCGTGGAGCACGAGTTCAGCAACCTGGAGAGAAGA 1260
                ** ** ******** ** ** ***** ** ***** ******   ** ** ** ******

HA              ATAGACAACCTAAACAAGAGAATGGAAGATGGATTTCTAGATGTGTGGACCTACAATGCT 1320
OptiHA          ATCGATAACCTGAACAAGAGAATGGAGGATGGCTTCCTGGATGTGTGGACCTACAACGCC 1320
                ** ** ***** ************** ***** ** ** ***************** ** 

HA              GAACTTTTAGTTCTACTGGAAAATGAGAGAACCTTGGACCTGCATGATGCCAATGTGAAG 1380
OptiHA          GAGCTGCTGGTGCTGCTGGAGAACGAGAGAACCCTGGATCTGCACGATGCCAACGTGAAG 1380
                ** **  * ** ** ***** ** ********* **** ***** ******** ******

HA              AACCTATATGAAAAAGTGAAATCACAATTGAGAGACAATGCAAAGGATTTGGGTAATGGG 1440
OptiHA          AACCTGTACGAGAAGGTGAAGAGCCAGCTGAGAGATAACGCCAAGGATCTGGGCAACGGC 1440
                ***** ** ** ** *****    **  ******* ** ** ****** **** ** ** 

HA              TGTTTTGAATTTTGGCATAAATGTGATGATGAATGCATCAACTCAGTTAAGAATGGCACG 1500
OptiHA          TGCTTCGAGTTCTGGCACAAGTGCGATGATGAGTGCATCAACAGCGTGAAGAACGGCACC 1500
                ** ** ** ** ***** ** ** ******** *********   ** ***** ***** 

HA              TATGACTACCCAAAATACCAAGACGAAAGCAAACTTAATAGGCAGGAAATAGATTCAGTG 1560
OptiHA          TACGATTACCCCAAGTACCAGGATGAGAGCAAGCTGAACAGACAGGAGATCGATAGCGTG 1560
                ** ** ***** ** ***** ** ** ***** ** ** ** ***** ** ***   ***

HA              AAACTGGAAAATCTGGGTGTATATCAAATCCTTGCTATTTATAGTACGGTATCGAGCAGT 1620
OptiHA          AAGCTGGAGAACCTGGGCGTGTACCAGATCCTGGCCATCTACAGCACCGTGAGCAGCAGC 1620
                ** ***** ** ***** ** ** ** ***** ** ** ** ** ** **   ***** 

HA              CTAGTTTTGGTGGGACTGGTCATTGCCATGGGTCTTTGGATGTGCTCGAATGGTTCAATG 1680
OptiHA          CTGGTGCTGGTGGGCCTGGTGATCGCCATGGGCCTGTGGATGTGCAGCAACGGCAGCATG 1680
                ** **  ******* ***** ** ******** ** *********   ** **   ***

HA              CAATGCAGGATATGTATATAA 1701
OptiHA          CAGTGCAGAATCTGCATCTRR 1701
                ** ***** ** ** ** *
```

* refers to the same nucleotide by alignment.

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