DEVELOPMENT AND ASSESSMENT OF NOVEL VACCINATION STRATEGIES FOR HEPATITIS B VIRUS INFECTION

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Australia

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To my loving family
for your boundless love, care, support and always being there for me

&

To Jean Ang
who stood by me when things looked bleak and supported me each step of the way
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Abstract

The current hepatitis B virus (HBV) vaccine is of no benefit in the treatment of patients with chronic HBV infection, and current antiviral therapies which inhibit the virus polymerase are not highly effective. The ultimate aim of this Ph.D. project was to develop and assess therapeutic vaccination strategies that induce immune responses that target virus infected hepatocytes and allow successful control of chronic HBV infection. To this end, a number of vaccination strategies were tested using the duck hepatitis B virus (DHBV) model. The DHBV model provides a versatile and reproducible experimental system for testing vaccination strategies as the outcomes of DHBV infection in ducks of different ages infected with different doses of virus have been well characterised.

In initial studies described in Chapter 3, recombinant DHBV core antigen (rDHBcAg) expressed in *E. coli*, was purified and used to immunise rabbits and mice to produce specific polyclonal and monoclonal antibodies for the detection of DHBV core antigen (DHBcAg). Immuno-staining techniques using these anti-DHBcAg-specific antibodies were then optimised. Immunoperoxidase detection of DHBcAg in duck liver sections was an essential part of this analysis as it allowed comparison with detection of DHBV surface antigen (DHBsAg) and confirmation of the percentage of DHBV-infected hepatocytes. Western Blot and immunofluorescent detection of DHBcAg were also developed and optimised and then all 3 immuno-staining techniques were used in subsequent Chapters to assess the efficacy of the different vaccination strategies.

As described in Chapter 4, duck CD40L (DuCD40L), was assessed as an immunological adjuvant in a protective DNA vaccine study. In humans, CD40L acts as a co-stimulatory molecule in the CD40-signalling pathway that is involved in the activation of antigen presenting cells (APC) and the generation of humoral and cell mediated immune (CMI)
responses. In the current studies the DuCD40L cDNA sequence was cloned using mRNA from duck peripheral blood mononuclear cells (PBMC) into the expression construct, pcDNA3, to yield pcDNA3-DuCD40L. Cells and supernatants of cells transfected with pcDNA3-DuCD40L were subsequently tested for bioactivity using in vitro assays. The cloning and analysis of expression of the DuCD40L was performed in our laboratory by Dr Feng Feng. The DuCD40L expression construct was then co-administered intramuscularly with DNA vaccines expressing the DHBV surface and core antigens to ducks at 14 and 28 days of age. Two weeks after the second vaccination, ducks were challenged intravenously (i.v.) with $4.5 \times 10^{10}$ DHBV genomes. Administration of the DuCD40L expression construct and the DHBV DNA vaccines in combination resulted in a 10-fold greater anti-DHBs antibody response and a significant decrease in the number of DHBV-infected hepatocytes at day 4 post-challenge (p.c.) compared to ducks that received DHBV DNA vaccines alone. Ultimately, as expected, all of the ducks successfully cleared their DHBV infection. Nevertheless, we determined that DuCD40L enhanced humoral immune responses and lead to reductions in the percentage of DHBV-infected hepatocytes following challenge.

As described in Chapter 5, the expression of DuCD40L was further assessed in a 2nd protective DNA vaccine study in 14-day-old ducks which are more susceptible to the development of persistent DHBV infection. The DuCD40L expression construct and DNA vaccines expressing the DHBV surface proteins were administered to ducks at 4 and 14 days of age. On the same day as the second vaccination, ducks were challenged i.v. with $5 \times 10^8$ DHBV genomes, a dose of DHBV that is 500-times higher than the dose known to result in persistent DHBV infection. Unexpectedly, following DHBV challenge, no significant differences in the percentage of DHBV-infected hepatocytes or anti-DHBs antibody titres were observed between ducks receiving DHBV DNA vaccines with DuCD40L expression construct and ducks receiving DHBV DNA vaccines alone. At day 21 p.c., all ducks vaccinated with DHBV DNA vaccines with or without the DuCD40L expression construct
had successfully cleared their DHBV infection while five out of five vector control ducks developed persistent DHBV infection. Interestingly, two out of five ducks that received the DuCD40L expression construct alone also cleared their DHBV infection. The studies described in Chapters 4 and 5 suggest DuCD40L may enhance immune responses in ducks and that DuCD40L should be further investigated as an immunological adjuvant in future vaccine studies.

Finally as described in Chapter 6, a post-exposure vaccination study was performed that combined treatment with the Bristol-Myers Squibb nucleoside analogue, Entecavir (ETV), and “prime-boost” vaccination using DNA vaccines and recombinant fowl-pox virus (rFPV) strains that express DHBV surface or core alone. Previous “prime-boost” vaccination studies in the laboratory had used DNA vaccines and rFPV strains that expressed both DHBV surface and DHBV core in combination. We aimed to determine if DHBV surface antigen which generates neutralising anti-DHBs antibodies, or DHBV core antigen which generates non-neutralising anti-DHBc antibodies, could provide the essential epitopes in a DNA vaccine and “prime-boost” protocol to enable the resolution of DHBV infection. 14 day-old ducks were inoculated i.v with $5 \times 10^8$ DHBV genomes and immediately treated with ETV (1.0mg/kg/day) for 14 days. At the same time, ducks received the “priming” DHBV DNA vaccines and 7 days later received the “boosting” vaccination with the rFPV-DHBV vaccines. The findings showed that protective humoral and CMI responses generated by “prime-boost” vaccination strategies with either DHBV surface or DHBV core alone blocked virus spread and replication and resulted in the targeting the destruction of infected hepatocytes and the resolution of DHBV infection. In contrast, ducks treated with ETV plus the control vectors showed restricted spread of DHBV infection in the liver during ETV treatment, but DHBV infection become widespread in four out of five ducks after ETV treatment was withdrawn. These findings indicate that DHBV surface and core antigen as measured in our studies are equally effective as components of our post-exposure “prime-boost” protocol. Since anti-
DHBc antibodies are non-neutralising this suggests that our “prime-boost” protocol may be generating effective CMI that were able to control DHBV infection.

The studies described in this thesis show that increasing levels of expression of DuCD40L may increase the efficacy of DNA vaccination protocols. Our novel vaccination strategy using ETV treatment in combination with “prime-boost” vaccination also suggests that DHBV surface and core antigen are both able to provide sufficient immunity to allow clearance of DHBV infection. Future studies are warranted to test other types of immunotherapy and antiviral agents to provide new directions for future therapeutic vaccination strategies for chronic HBV infection.
Declaration of originality and consent

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Devin TEOH

1st November 2014
Publications, presentations and awards arising from this thesis

Manuscripts published


Manuscripts in preparation

C.Q. Teoh, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. “Entecavir therapy combined with “prime-boost” vaccination with either duck hepatitis B virus (DHBV) surface or core are able to block virus replication and spread and target DHBV-infected hepatocytes.”

C.Q. Teoh, F. Feng, Q. Qiao, B. Baradaran, A.R. Jilbert. “Cloning, expression and use of a duck CD40 ligand (CD40L) DNA construct as a molecular adjuvant for duck hepatitis B virus DNA vaccines.”

C.Q. Teoh, F. Feng, A.R. Jilbert. “Purification of recombinant duck hepatitis B core antigen (rDHBcAg) particles for production of specific polyclonal and monoclonal antibodies against DHBcAg.”
Oral presentations


**C.Q. Teoh**, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. Entecavir therapy combined with “prime-boost” vaccination with either duck hepatitis B virus (DHBV) surface or core are able to block virus replication and spread and target DHBV-infected hepatocytes. Australian Centre for HIV and Hepatitis Virology Workshop, Novotel Barossa Valley Resort, June, 2008.


Poster presentation


**C.Q. Teoh**, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. Entecavir therapy combined with “prime-boost” vaccination with either duck hepatitis B virus (DHBV) surface
or core are able to block virus replication and spread and target DHBV-infected hepatocytes.

14th International Congress of Immunology (ICI), Kobe, Japan, August, 2010.


C.Q. Teoh, F. Feng, D. Boyle, R. Colonno, B. Baradaran, A.R. Jilbert. Entecavir therapy combined with “prime-boost” vaccination with either duck hepatitis B virus (DHBV) surface or core are able to block virus replication and spread and target DHBV-infected hepatocytes. University of Adelaide, School of Molecular and Biomedical Science, Research Symposium, National Wine Centre, Adelaide, December, 2008.

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Australian Society of Microbiology (ASM) SA Branch Student Research Award, 2010.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>~</td>
<td>Approximately</td>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
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<tr>
<td>1H.1</td>
<td>Monoclonal anti-pre-S/S antibodies</td>
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<tr>
<td>3TC</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AB</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ADV</td>
<td>Adefovir</td>
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<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>Amp</td>
<td>Ampicilin</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>APV</td>
<td><em>Avipoxvirus</em></td>
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<tr>
<td>ASHV</td>
<td>Arctic squirrel hepatitis virus</td>
</tr>
<tr>
<td>AusDHBV</td>
<td>Australian strain of DHBV</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Core</td>
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<tr>
<td>cccDNA</td>
<td>Covalently closed circular DNA</td>
</tr>
<tr>
<td>CD154</td>
<td>CD40 ligand</td>
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<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CESC</td>
<td>Chicken embryonic skin cells</td>
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<td>CHB</td>
<td>Chronic HBV infection</td>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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CMI  Cell-mediated immunity
ConA  Concanavalin A
C-ORF  Core open reading frame
CPE  Cytopathic effect
CpG  Cytosine-phosphate-guanosine
CQT-1  Polyclonal rabbit anti-DHBc antibodies
CQT-2  Polyclonal rabbit anti-DHBc antibodies
CsCl  Caesium chloride
CSIRO  Commonwealth Scientific and Industrial Research Organisation
CTL  Cytotoxic T lymphocytes
DAB  3,3-diaminobenzidine tetrahydrochloride
DAPI  4',6-diamidino-2-phenylindole
DCs  Dendritic cells
dGTP  Deoxyguanosine triphosphate
DHBcAg  DHBV core antigen
DHBcAg  DHBV e antigen
DHBV  Duck hepatitis B virus
DMEM  Dulbecco’s modified Eagle’s minimal essential
dNTP  Deoxynucleotide triphosphates
dsDNA  Double-stranded linear virus DNA
DuCD40L  Duck CD40 ligand
DW  Distilled water
E. coli  Escherichia coli
EAA  Ethanol: Acetic Acid
EDTA  Ethylenediaminetetraacetic acid
EEV  Extracellular enveloped virus
ELISA  The enzyme-linked immunosorbent assay
<table>
<thead>
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<tr>
<td>ELISPOT</td>
<td>The enzyme-linked immunosorbent spot assay</td>
</tr>
<tr>
<td>ETV</td>
<td>Entecavir</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund’s complete adjuvant</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FPV</td>
<td>Fowl pox virus</td>
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<tr>
<td>G</td>
<td>Gauge</td>
</tr>
<tr>
<td>gm</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>Centrifugal force</td>
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<tr>
<td>GSHV</td>
<td>Ground squirrel hepatitis virus</td>
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<tr>
<td>HBcAg</td>
<td>HBV core antigen</td>
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<td>HBeAg</td>
<td>HBV e antigen</td>
</tr>
<tr>
<td>HBsAg</td>
<td>HBV surface antigen</td>
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<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>IF assay</td>
<td>Immuno-fluorescence assay</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
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<td>IFN-g</td>
<td>Interferon-gamma</td>
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<td>IFN-α</td>
<td>Interferon-alfa</td>
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<tr>
<td>IFN-α2A</td>
<td>Interferon-alfa-2a</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>IFN-β</td>
<td>Interferon-beta</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgY</td>
<td>Immunoglobulin Y</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IMV</td>
<td>Intracellular mature virus</td>
</tr>
<tr>
<td>IMVS</td>
<td>Institute of Medical and Veterinary Science</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>ITRs</td>
<td>Identical inverted terminal repeats</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LMH</td>
<td>Chicken hepatoma cell line</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MAbSA</td>
<td>Monoclonal Antibodies SA</td>
</tr>
<tr>
<td>MHC-I</td>
<td>Class I histocompatibility molecules</td>
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<tr>
<td>MHC-II</td>
<td>Class II histocompatibility molecules</td>
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<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MVA</td>
<td>Modified vaccinia Ankara</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleot(s)ide analogues</td>
</tr>
<tr>
<td>NC membrane</td>
<td>Nitrocellulose membrane</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal duck serum</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NK-T</td>
<td>Natural Killer T-cell</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
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<td>NMS</td>
<td>Normal mouse serum</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complexes</td>
</tr>
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<td>NRS</td>
<td>Normal rabbit serum</td>
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<tr>
<td>NSS</td>
<td>Normal sheep serum</td>
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<tr>
<td>NTCP</td>
<td>Sodium taurocholate co-transporting polypeptide</td>
</tr>
<tr>
<td>O/N</td>
<td>Over night</td>
</tr>
<tr>
<td>OPD</td>
<td>O-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>p</td>
<td>p-value</td>
</tr>
<tr>
<td>p.c.</td>
<td>Post challenge</td>
</tr>
<tr>
<td>p.f.u</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post-infection</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline-Tween</td>
</tr>
<tr>
<td>PCEF</td>
<td>Primary chicken embryonic fibroblasts</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDEF</td>
<td>Primary duck embryonic fibroblasts</td>
</tr>
<tr>
<td>Peg</td>
<td>Pegylated</td>
</tr>
<tr>
<td>Pg-RNA</td>
<td>Pre-genomic RNA</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin M</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
</tbody>
</table>
P-ORF  Polymerase open reading frame
PreC  Pre-core
pre-S/S  Pre-surface
PS  Pre-surface
PRR  Pattern recognition receptors
qPCR  Quantitative PCR
qRT-PCR  Quantitative reverse transcription PCR
R39408  Polyclonal rabbit anti-DHBc antibodies
rcDNA  Relaxed circular DNA
RE  Restriction Endonuclease
rFPV  Recombinant FPV
rFPV-124  rFPV-DHBV strains expressing DHBV core
rFPV-125  rFPV-DHBV strains expressing pre-surface antigens
rFPV-DHBc  rFPV-DHBV strains expressing DHBV core
rFPV-DHBpre-S/S  rFPV-DHBV strains expressing pre-surface antigens
rFPV-DHBV  rFPV strains expressing DHBV antigens
RGHBV  Ross’ goose hepatitis B virus
rHBsAg  Recombinant HBsAg
RI DNA  Replicative intermediate DNA
RT  Room temperature
S  Surface
s.c.  Subcutaneous
SDS  Sodium dodecyl sulphate
Sec  Second
SGHBV  Snow goose hepatitis virus
SOC medium  Super optimal culture medium
S-ORF  Surface open reading frame
TCR          T cell receptor
TEMED        Tetramethylethylenediamine
Tet          Tetracycline
TFV          Tenofovir
Th1          Type I
TLR          Toll-like receptors
TNF-α        Tumour necrosis factor-alpha
USA          United States of America
V            Volts
v.g.e.       Virus genome equivalents
WHO          World Health Organisation
WHV          Woodchuck hepatitis virus
WMHBV        Woolly monkey hepatitis B virus
X            X protein
X-ORF        X protein open reading frame
Chapter 1 - Introduction

1.1 Background: Epidemiology of HBV infection

Hepatitis B virus (HBV) infection is a common, often life-long and highly prevalent infectious disease worldwide. The human HBV is a highly infectious DNA virus that is able to remain on any surface such as razor blades, blood stains for about a week without losing its infectivity (WHO, 2002). HBV is transmitted by exposure to infected serum or body fluids such as semen and vaginal fluid and by horizontal routes that include sexual intercourse, needle stick injuries, blood transfusion and organ transplantation. Another major route of infection is vertical transmission from the infected mother to the infant at the time of birth (Lok and McMahon, 2001; CDC, 2008). Chronic HBV infection (CHB) has a lower prevalence in developed countries (e.g. Western countries) where infection mainly occurs through horizontal transmission. In contrast CHB has a higher prevalence in developing countries, e.g. China, Southeast Asia and tropical Africa where vertical transmission is more common (Grimm et al., 2011).

HBV infection is a major health care problem with 2 billion people alive today having been infected with HBV at some time in their life (Huang et al., 2011). Despite the widespread use of prophylactic vaccines against HBV, new infections continue to occur and it is estimated that ~350-400 million people worldwide have CHB (Block et al., 2007; Grimm et al., 2011; Woo et al., 2010).
1.2 Hepadnaviruses

1.2.1 Hepadnaviridae Family
The Hepadnaviridae family is a family of hepatotropic viruses that infect the liver and cause liver disease in mammals and birds. Hepadnaviruses can be subdivided into 2 genera: members of the *Orthohepadnavirus* genus infect mammals and include the human HBV, the woodchuck hepatitis virus (WHV), the woolly monkey hepatitis B virus (WMHBV), the arctic squirrel hepatitis virus (ASHV) and the ground squirrel hepatitis virus (GSHV). Similarly, members of the *Avihepadnavirus* genus infect avian species and include the duck hepatitis B virus (DHBV), the heron hepatitis B virus (HHBV), the snow goose hepatitis B virus (SGHBV) and the Ross’ goose hepatitis B virus (RGGHBV) (Table 1.1). All Hepadnaviruses share similar structural features and replication strategies and each has a high degree of species specificity. However, some Hepadnaviruses can infect closely related species, e.g. HBV is infectious to chimpanzees, which provides the most closely related animal model to study human HBV (Funk *et al.*, 2007; Zoulim *et al.*, 2008).

1.2.2 Hepatitis B virus structure and genome organisation
The HBV genome is ~3182 base pairs (bp) in length and is a partially double-stranded relaxed circular DNA (rcDNA) molecule. The diameter of the HBV virion is approximately 42-47 nm with a double-shelled structure (Kann, 2002; Skrastina *et al.*, 2008).

The HBV DNA genome contains four open reading frames (ORF) all located on the negative DNA strand which include the core ORF (C-ORF), the surface ORF (S-ORF), the X-ORF and the polymerase ORF (P-ORF) (Figure 1.1A). These four ORF are partially overlapping, with the P-ORF overlapping the other three ORF and spanning approximately two thirds of the HBV genome (Karayiannis, 2003).
Table 1.1: Members of the hepadnaviridae

<table>
<thead>
<tr>
<th>Orthohepadnaviruses</th>
<th>Avihepadnaviruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis B virus (HBV)(^a)</td>
<td>Duck hepatitis B virus (DHBV)(^a,k,c)</td>
</tr>
<tr>
<td>Woodchuck hepatitis virus (WHV)(^a,d)</td>
<td>Heron hepatitis B virus (HHBV)(^e,c)</td>
</tr>
<tr>
<td>Ground squirrel hepatitis virus (GSHV)(^f,g)</td>
<td>Snow goose hepatitis virus (SGHBV)(^h,c)</td>
</tr>
<tr>
<td>Arctic squirrel hepatitis virus (ASHV)(^i)</td>
<td>Ross’ goose hepatitis B virus (RGGHBV)(^h,c)</td>
</tr>
<tr>
<td>Woolly monkey hepatitis B virus(^j) (WMHBV)</td>
<td>Crane hepatitis B virus (CHBV)(^k,c)</td>
</tr>
<tr>
<td></td>
<td>Stork hepatitis B virus (STHBV)(^l,c)</td>
</tr>
</tbody>
</table>

\(^a\)(Mason et al., 1980)  
\(^b\)(Triyatni et al., 2001)  
\(^c\)(Funk et al., 2007)  
\(^d\)(Galibert et al., 1982)  
\(^e\)(Sprengel et al., 1988)  
\(^f\)(Marion et al., 1980)  
\(^g\)(Seeger et al., 1984)  
\(^h\)(Chang et al., 1999)  
\(^i\)(Testut et al., 1996)  
\(^j\)(Landford et al., 1998)  
\(^k\)(Prassolov et al., 2003)  
\(^l\)(Pult et al., 2001)
**Figure 1.1:** Diagrammatic representation of the hepadnavirus genome structure. In both the HBV and DHBV genomes, the outer blue lines represent the RNA transcripts. The inner black lines represent the full-length negative strand and the incomplete positive strand of the virus DNA. The full-length negative strand has the viral polymerase covalently attached to its 5’ end. Its positive-strand has a RNA primer at the 5’ end, and is partially complete at the 3’ end (represented by the dotted lines). during virus replication, pg-RNA is reversed transcribed and then digested by RNaseH to produce RI DNA and rcDNA The S-ORF, the P-ORF, the C-ORF and the X-ORF are shown in coloured lines and all located on the negative DNA strand.

**Panel A:** HBV genome structure

The HBV genome consists of four distinct overlapping ORF that encode for seven viral proteins. They are L, M, S of HBsAg by the S-ORF; polymerase by the P-ORF; HBcAg and HBeAg by the C-ORF and HBx by the X-ORF. Obtained from Beck and Nassal, 2007 with permission from the World Journal of Gastroenterology.

**Panel B:** DHBV genome structure (GenBank # K01834)

The DHBV genome consists of four distinct overlapping ORF that encode for five viral proteins. They are DHBpre-S/S and DHBsAg by the S-ORF; polymerase by the P-ORF; DHBcAg and DHBcAg by the C-ORF. The X-ORF is shown but there is no consensus as to whether the X-gene exists in DHBV. Obtained from Funk et al., 2007 with permission from the World Journal of Gastroenterology.
The C-ORF has two in-frame start codons that code for the HBV core protein also referred to as core antigen (HBcAg) and the HBV e protein also referred to as e antigen (HBeAg). Dimerisation of the HBcAg protein is required for the formation of the ~36 nm icosahedral nucleocapsid that contains the HBV genome. The HBeAg also known as pre-core (PreC) protein, is a secreted form of HBcAg (Seeger and Mason, 2000; Bruss, 2004). In CHB, HBeAg in the serum is an indicator of virus replication, and sero-conversion from HBeAg-positive to anti-HBe antibodies is associated with a decrease in levels of virus replication and improved liver histology (Lai and Liaw, 2010) as discussed in Section 1.4.3.

The S-ORF of HBV contains the Pre-S1, Pre-S2 and S genes, that encode the large (L), medium (M), and small (S) HBV surface antigen (HBsAg) proteins respectively (Figure 1.2A). These proteins are embedded in host-derived lipid and are involved in the formation of the viral envelope (Karayiannis, 2003). The S protein contains an epitope known as the “a” determinant, which is important in eliciting production of neutralising anti-HBs antibodies (Cooreman et al., 2001). The S protein comprises ~70% of the HBsAg proteins present in the envelope of HBV virions, while the remaining 30% is comprised of M and L proteins in roughly equal amounts. S and M proteins also form ~22 nm non-infectious spherical and filamentous HBsAg particles that circulate in the bloodstream in at least 100-fold greater number than HBV virions. These spherical and filamentous HBsAg particles do not contain a nucleocapsid. However, formation of the complexes between these non-infectious HBsAg particles and neutralising antibodies targeting HBsAg (anti-HBs antibodies) suggests that excessive serum HBsAg may function as a tolerogen, assisting virus transmission and persistence during CHB (Seeger and Mason, 2000; Glebe and Urban, 2007; Skrastina et al., 2008).

The P-ORF encodes the virus polymerase (Pol), a large multifunctional protein that has terminal protein, RNase H and reverse transcriptase (RT) activities. As described in Section
Figure 1.2: Schematic representation of the structure of the hepadnaviral virion

Panel A: The HBV virion with a 3182 bp genome (GenBank # NC_003977)


Panel B: The DHBV virion showing a 3027 bp genome (GenBank # AJ006350)

Adapted from Jilbert and Kotlarski, 2000 with permission from Developmental & Comparative Immunology.
A
Envelope or surface proteins
L-HBsAg
M-HBsAg
S-HBsAg
HBV
3182 bp
Polymerase
Nucleocapsid or core protein

B
Envelope or surface proteins
PreS/S protein
S protein
DHBV
3027 bp
Polymerase
Nucleocapsid or core protein
1.3.1, during virus replication, pre-genomic RNA (pg-RNA) is reversed transcribed and then digested by RNaseH to produce replicative intermediate DNA (RI DNA) and rcDNA (Summers and Mason, 1982; Kim et al., 1999; Lanford et al., 1995).

The X-ORF of HBV encodes the X protein (HBx) and little is known about its function in natural infection (Kann, 2008). A recent study using primary human hepatocytes infected with either wild-type HBV or HBx deficient HBV has demonstrated that HBx is required to maintain productive HBV infection. The study suggests that HBx initiates and maintains transcription from covalently closed circular DNA (cccDNA) by controlling histone acetylation (Lucifora et al., 2011).

1.2.3 Duck hepatitis B virus structure and genome organisation

DHBV shares similar structure and genome organisation to HBV (Mason et al., 1980). The DHBV DNA genome is ~ 3021-3027 bp in length and is partially double-stranded DNA (Triyatni et al., 1998). DHBV virions are 40-45 nm in diameter (Franke et al., 2007).

The DHBV genome only contains three ORF, as there is no consensus on whether the X-gene exists in DHBV (Funk et al., 2007) (Figure 1.1B). These three ORF are C-ORF, S-ORF and P-ORF encoding the DHBV core, surface and polymerase proteins respectively. The C-ORF encodes the secreted DHBV e (DHBeAg) protein, and the DHBV core (DHBcAg) protein that forms the icosahedral nucleocapsid (Funk et al., 2007). The S-ORF encodes two surface antigen (DHBsAg) proteins, namely S and pre-S/S DHBV surface antigen (Funk et al., 2007) (Figure 1.2B). The pre-S/S protein binds to carboxypeptidase D (gp180), a cellular receptor on hepatocytes, to initiate virus entry and then DHBV infection (Spanhenberg et al., 2001; Breiner et al., 1998). The serum of DHBV-infected ducks also contains non-infectious DHBsAg particles. These particles are found at ~500-1000 fold excess to DHBV virions and are found as roughly spherical forms with a diameter of 40-45 nm. Unlike HBV, DHBsAg
particles are not found as filaments or small spheres (Franke et al., 2007). The P-ORF encodes the DHBV polymerase that has RNA- and DNA-dependent DNA polymerase and RNase H activities (Jilbert and Kotlarski, 2000).

1.3 Hepadnavirus Replication

1.3.1 Viral entry, transcription and translation to generate new virus

Hepadnaviruses primarily infect and replicate in hepatocytes, the major cell-type in the liver. All members of this family replicate via reverse transcription of an RNA intermediate. Hepadnaviruses facilitate the attachment to susceptible cells by using Pre-S1 in HBV (Neurath et al., 1986) and Pre-S/S protein in DHBV as a ligand followed by penetration of the cell surface (Spanhenberg et al., 2001; Breiner et al., 1998). By using near zero distant photocross-linking and tandem affinity purification, a recent study demonstrated that sodium taurocholate co-transporting polypeptide (NTCP), a multiple transmembrane transporter predominantly expressed in the liver is a functional receptor for human HBV (Yan et al., 2012). During the initiation of infection, rcDNA is transported to the nucleus and is converted to cccDNA, a stable virus mini chromosome (Bock et al., 2001). In the nucleus of infected hepatocytes, cccDNA serves as a template for transcription by host RNA polymerase II of pg-RNA and mRNA. The pg-RNA and the Pol are then packaged into the immature nucleocapsid where pg-RNA is reverse transcribed through RI DNA to produce rcDNA and double-stranded linear virus DNA (dsDNA) in ~10:1 ratio. Then nucleocapsids that contain the newly made rcDNA or dsDNA either gain an outer envelope by budding in the ER and are released as progeny virus or reenter the nucleus to increase the copy number of cccDNA (Figure 1.3) (Seeger and Mason, 2000; Beck and Nassal, 2007; Nassal, 2008).
Figure 1.3: Schematic diagram showing the different stages of hepadnavirus life cycle during replication.

Adapted from Jilbert and Kotlarski, 2000 with permission from Developmental & Comparative Immunology.

Major steps of the HBV replication cycle are shown. After attachment and translocation of the virion DNA to the nucleus, rcDNA is converted to supercoiled cccDNA that forms a stable virus mini chromosome. The cccDNA is then transcribed to produce pg-RNA and mRNAs, followed by protein production, assembly of nucleocapsids and packaging of pg-RNA. Within capsids, reverse transcription of pg-RNA through RI DNA occurs to produce rcDNA and dslDNA genome in ~ 10:1 ratio. Newly made rcDNA and dslDNA are enveloped and exported as progeny virus, or can return to the nucleus by way of an intracellular pathway, which results in an amplification of cccDNA, typically found at 30-50 copies per hepatocyte.
Progeny virus

Intracellular amplification of cccDNA

cccDNA

Integration (rare)

~90%

~10%

rcDNA

DSL DNA

Progeny virus
1.3.2 An important reservoir of hepadnavirus infection – cccDNA

During infection, hepadnaviruses accumulate 10-50 copies of cccDNA in the nucleus of the infected hepatocytes (Summers et al., 1990). Current antiviral drugs have been shown to be unable to eradicate cccDNA (Beck and Nassal, 2007; Grimm et al., 2011). A study that was conducted in non-dividing hepatocyte cultures has shown that WHV cccDNA has a half-life of more than 30 days (Moraleda et al., 1997). Therefore, elimination of the cccDNA reservoir from infected hepatocytes is thought to be important in the resolution of chronic HBV infection (Block et al., 2007). Two immune-mediated mechanisms have been suggested to be essential for the elimination or clearance of cccDNA: 1) A cytolytic mechanism that involves cytotoxic T lymphocytes (CTL)-mediated killing of infected hepatocytes that contain cccDNA and 2) a non-cytolytic mechanism that involves “curing” of infected hepatocytes by cytokines. These cytolytic and non-cytolytic mechanisms will be discussed in Section 1.4.2.2.

1.4 Outcomes of HBV infection

HBV infection itself is non-cytopathic. It is the effectiveness of host immune response which determines the associated liver diseases and the outcomes of the infection, which can be either acute or chronic (McMahon, 2005) (Figure 1.4). These different outcomes are determined by the effectiveness of the host immune responses as described in Section 1.4.1.1 and 1.4.2.1. The outcomes of HBV infection are also affected by the virus dose and the age of the host at the time of virus infection. For example, 90% of infants infected with HBV at birth and 25-50% of children infected with HBV at 1-5 years of age develop chronic HBV infection compared with only ~5% of adults infected with HBV (Sprengers and Janssen, 2005; Villeneuve, 2005).

The serological markers detected during acute and chronic HBV infection are summarised in Table 1.2.
**Figure 1.4:** Serological profile of virological and immunological markers following acute (Panel A) and chronic HBV infection (Panel B).

Obtained from Bowden 2008 with permission from International Medical Press.

**Panel A:** Acute HBV infection

HBV DNA, HBsAg, HBeAg and anti-HBc antibodies are detected in the serum after infection. Resolution of infection occurs with the appearance of protective anti-HBs antibodies and a fall in levels of HBV DNA and HBsAg to below the levels of detectability. Seroconversion from HBeAg to anti-HBe antibodies happen after ~ 14 weeks. Anti-HBc antibodies are a marker of infection and persist for many years.

**Panel B:** Chronic HBV infection

High titres of HBV DNA, HBsAg, HBeAg and anti-HBc antibodies are detected in the serum for many years. The protective anti-HBs antibodies are not detected in chronic HBV infection. Spontaneous seroconversion of HBeAg to anti-HBe antibodies occurs at a rate of 10% per year in chronic HBV infection. Patients who undergo HBeAg seroconversion have a good prognosis but this does not represent resolution of HBV infection.
Table 1.2: Serological markers during HBV infection

<table>
<thead>
<tr>
<th>Status</th>
<th>HBsAg</th>
<th>Anti-HBs</th>
<th>Anti-HBc</th>
<th>HBeAg</th>
<th>Anti-HBe</th>
<th>HBV viral load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Low</td>
</tr>
<tr>
<td>Acute</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>High</td>
</tr>
<tr>
<td>Recovery</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Chronic (&lt;6 months)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>High</td>
</tr>
<tr>
<td>Chronic (&gt;6 months)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>High or Low</td>
</tr>
<tr>
<td>Immunisation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Undetectable</td>
</tr>
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</table>
1.4.1 Acute HBV infection

In acute HBV infection, ~1% of infections result in fatal fulminant hepatic failure. Acute HBV infection has an initial 2 to 6 weeks incubation period, followed by a flare of the alanine aminotransferase (ALT) level and an increase in HBV DNA and HBsAg levels in serum. The anti-HBc antibody response is first detected in the serum, ~2 weeks after HBsAg is detected. Subsequently, the resolution of an acute HBV infection is characterized by the normalization of the ALT levels, loss of HBV DNA and HBsAg from the serum, and the seroconversion of the HBsAg to anti-HBs antibodies. Another important serological marker is anti-HBe antibodies; seroconversion from HBeAg to anti-HBe antibodies indicates a slower virus replication rate and is usually observed during the resolution of HBV infection. Most of the individuals who recover from HBV infection are disease and HCC free. However, traces of HBV DNA may persist for life in the liver, and can contribute to the reactivation of HBV infection (Petit et al., 2001; Villeneuve, 2005).

1.4.1.1 Resolution of acute HBV infection

The mechanism of resolution of HBV infection is not well understood. It has been suggested in addition to innate immune responses, strong cell mediated immunity (CMI) and humoral immune responses toward HBsAg, HBeAg, HBx and Pol are responsible for the resolution acute HBV infection (Kara et al., 2004).

The innate immune response is believed to be the first-line host defence mechanism during HBV infection but is not well studied. Immediately after virus infection, the presence of viral RNA or DNA triggers the production of interferon (IFN)-α/β cytokines (Bertoletti and Gehring, 2006). The production of interferon (IFN)-α/β cytokines may enhance the expression of class I histocompatibility molecules (MHC-I) on infected cells and therefore may increase their susceptibility to CTL attack. Interestingly, production of IFN-α is not
detected during the early phase of acute HBV infection in chimpanzees suggesting that HBV might have evolved an evasion strategy to escape from the early host immune response (Wieland et al., 2004). Another component of the innate immune response is natural killer (NK) cells and NK-T-lymphocytes which use either a cytolytic or non-cytolytic mechanism to control virus infection. However, the role of these cells in HBV clearance is not well characterised (Chang and Lewin, 2007; Scully et al., 1990).

CMI provides no protection against initial virus infection but is important in the destruction of virus infected hepatocytes. After infection, presentation of endogenous/exogenous HBV antigens by antigen presenting cells (APC) to T-lymphocytes results in the activation of HBV-specific CD4+ and CD8+ T-lymphocytes, also known as CTL. Patients with acute HBV infection are characterised by detectable HBV-specific CD4+ and CD8+ T responses with a type I (Th1) profile of cytokine production (Bertoletti et al., 2006). CD4+ helper T lymphocytes play an important role in resolution of chronic HBV infection. CD4+ helper T lymphocytes not only facilitate the antibody production by B lymphocytes but also facilitate the induction and maintenance of HBV antigen specific CTL.

CTL are the major cellular subset responsible for clearance of HBV-infected hepatocytes and for reducing the levels of circulating virus through cytolytic and non-cytolytic mechanisms: the cytolytic pathway induces apoptosis of infected hepatocytes. The elimination of existing cccDNA in infected hepatocytes prevents the spread of HBV to other susceptible hepatocytes (Thimme et al., 2003; Mizukoshi et al., 2004; Bertoletti and Gehring 2006). When the infected hepatocytes are killed or naturally die, hepatocytes are replaced by an uninfected lineage or by neighbouring infected hepatocytes. During cell division, there are two possible fates for cccDNA in the infected hepatocytes: (1) the cccDNA is released in the cytoplasm when nuclear membrane breakdown during mitosis and is subsequently lost by degradation. This results in virus-free progeny hepatocytes. (2) The cccDNA may survive during mitosis
for example by tethering to host chromosomes and therefore the complete loss of cccDNA from infected hepatocytes requires more rounds of hepatocyte turnover (Zoulim, 2005; Mason et al., 2007; Levrero et al., 2009).

The non-cytolytic mechanism involves a wide range of anti-viral cytokines including IFN-α, IFN-γ and tumour necrosis factor-alpha (TNF-α) to de-activate the intracellular viruses (Guidotti and Chisari, 2001; Guidotti, 2002). Studies have shown that IFN-γ and TNF-α suppress HBV replication by preventing the assembly of the HBV pg-RNA-containing nucleocapsids through the NF-kappa B pathway, and degradation of viral RNA and proteins via proteasome- and kinase-dependent activity (Biermer et al., 2003; Chang and Lewin, 2007; Chisari et al., 2010). In addition, in a study by Schulz et al., 1999, duck IFN-γ inhibited DHBV replication non-cytopathically in infected PDH by eliminating nucleocapsids containing pg-RNA, but had no effect on early virus replication steps as initial cccDNA levels were not decreased in IFN-γ treated cells. All these findings support the existence and the importance of the non-cytolytic mechanisms.

Another adaptive immune response that plays an important role in the resolution of acute HBV infection is the humoral immune response characterized by production of antiviral antibodies. The antibodies such as anti-HBs antibodies are able to neutralise free virus particles and can prevent (re)infection. Anti-HBs antibodies bind to the HBV envelope: (1) to inhibit the interaction between the virus and hepatocytes and (2) to facilitate the clearance of virus from the blood by opsonising virus particles to allow the process of phagocytosis (Akbar et al., 1999). Anti-HBs antibodies are a marker of resolution in acute HBV infection and provide lifetime protection against HBV re-infection (Akbar et al., 1999; Chang and Lewin, 2007). In contrast, anti-HBc antibodies are non-neutralising antibodies that serve as a marker for current or past HBV infection and do not have any protective ability (Menne and Tennant,
1999; Seeger and Mason, 2000; CDC, 2011). In addition, the antiviral response of antibody-dependent cell-mediated cytotoxicity (ADCC), a mechanism mainly mediated by NK cells by targeting cells that have been bound by specific antibodies, may also be important in HBV infection (Gonzales et al., 1979).

1.4.2 Chronic HBV infection

Chronic HBV infection is defined as the presence of HBsAg and HBV DNA in the serum for more than six months (Bertoletti and Ferrari, 2003). Each year, sero-conversion to anti-HBs antibodies occurs in ~1% of HBV carriers (Manno et al., 2004). In CHB, active virus replication occurs throughout the course of infection with weak or undetectable HBV-specific T cell immune responses (Rehermann et al., 1996). However, these weak immune responses are sufficient to cause progressive liver damage. Chronic HBV infection can be viewed in four stages: immune tolerance, immune clearance, immune control and reactivation (Figure 1.5). However, not all chronically HBV infected individuals experience all of the phases (Yim and Lok, 2006; Kwon and Lok, 2011).

The first stage (immune tolerance stage) occurs most commonly in individuals with perinatally acquired infections. In contrast, an immune tolerance phase is rarely observed in individuals who acquired HBV infection during childhood or adulthood (Damme et al., 2013). In this stage, HBV replicates actively but host immune responses to the virus are minimal. This stage is characterized by high levels of HBV DNA and HBsAg in serum, the presence of HBeAg and normal ALT levels (Villeneuve, 2005; Yim and Lok, 2006). A normal ALT level indicates minimal or no inflammation in the liver during the immune tolerance phase that can last for up to 10 years. Immune-mediated killing of hepatocytes is less likely in this stage suggesting that clonal deletion of HBV-specific T cells has occurred in these individuals (Huang et al., 2011).
The second stage (immune clearance stage) occurs during adolescent or adult life. This stage is characterized by the presence of HBeAg, fluctuations in HBV DNA levels and a rise in ALT levels. A high level of ALT indicates host immune response to HBV is activated and leads to a constant attack on infected hepatocytes, causing liver damage and inflammation (Villeneuve, 2005; Yim and Lok, 2006). Notably, spontaneous seroconversion of serum HBeAg to anti-HBe antibodies occurs at a rate of 10% per year in this stage (Huang et al., 2011). Prolonged presence of HBeAg and high levels of HBV DNA are associated with cirrhosis and a higher risk of HCC. Therefore, patients who undergo HBeAg seroconversion have a good prognosis (Ferir et al., 2008; Huang et al., 2011; Kwon and Lok, 2011).

The third stage (inactive carrier or immune control stage) is characterized by low levels of HBV DNA and HBsAg in the serum, the absence of HBeAg, the presence of anti-HBe antibodies, and normal ALT levels. In this stage, minimum or mild inflammation in the liver may persist. However, it can be reversed to the immune clearance stage during immunosuppression or after liver transplantation (Villeneuve, 2005; Yim and Lok, 2006). This could be due to the presence of residual HBV cccDNA, which has been found to persist at low levels in the liver even after resolution of acute HBV infection (Hu, 2002).

The last stage (reactivation stage) occurs during the later stages of life, usually manifest in older patients with advanced liver disease. This stage is characterized by the absence of HBeAg, the presence of anti-HBe antibodies, fluctuations in HBV DNA and ALT levels and active inflammation in the liver due to the reactivation of virus replication. Furthermore, many patients in this stage have HBeAg-negative mutants (precore or core promoter HBV mutants) that can prevent or reduce the production of HBeAg (Villeneuve, 2005; Yim and Lok, 2006; Kwon and Lok, 2011).
Figure 1.5: Natural history illustrating the difference phases of CHB infection

Chronic HBV infection can be viewed in four stages: immune tolerance, immune clearance, inactive carrier (or immune control) and reactivation stage. ALT levels may fluctuate or be increased during the immune clearance or reactivation stage. HBV DNA levels are high in the immune tolerance stage and decreased during immune clearance and inactive carrier stages. In the reactivation stage, HBV DNA levels may fluctuate. Spontaneous seroconversion of HBeAg to anti-HBe antibodies occurs at a rate of 10% per year during the immune clearance stage. Obtained from Kwon and Lok, 2011 with permission from Nature Reviews Gastroenterology and Hepatology.
1.5 Animal model – Duck Hepatitis B Virus

The study of HBV infection can only be conducted in chimpanzees or through naturally established HBV infection in humans and these studies are limited by cost, ethical considerations and the availability of subjects. Most HBV research is conducted in animal models by studying related hepadnavirus infections in their natural hosts to gain a better understanding of the virological and immunological responses that can be correlated to acute and chronic HBV infection (Wieland and Chisari, 2005). DHBV-infected ducks and WHV-infected woodchucks provide versatile animal models for the study of hepadnavirus infection (Karayiannis, 2003). The DHBV model is a widely accepted hepadnavirus animal model. DHBV shares ~ 40% genome homology with, and has similar genome organisation to, HBV. DHBV infection outcomes are viral dose and host age dependent, as is seen in HBV infection in humans (Jilbert et al., 1998). These characteristics make the DHBV-infected ducks a suitable hepadnavirus animal model in which to study the efficacy and response to anti-viral treatments and vaccines and outcomes of DHBV infection.

1.6 Outcomes of DHBV infection

As in HBV infection, the outcome of DHBV infection is dependent on host age, viral dose and the effectiveness of the host immune response (Jilbert et al., 1998; Foster et al., 2005). Jilbert et al., 1998 have shown that with the same virus dose, younger ducks are more likely to develop persistent DHBV infection than older ducks. In the same study, ducks of the same age were given different doses of virus, resulting in either acute or persistent DHBV infection, with ducks given the largest dose more prone to develop persistent DHBV infection (Figure 1.6). In conclusion, a large infective dose, younger ducks and weaker immune responses tend to result in persistent DHBV infection.
**Figure 1.6:** Dose and age-related relationship of infection outcome in DHBV-infected ducks

This curve is constructed to determine the minimum dose of DHBV that can establish persistent DHBV infection in ducks of various ages. Inoculation of DHBV with doses above the red line resulted in persistent DHBV infection; In contrast, doses below the red line result in acute/resolved DHBV infection.

(Adapted from Jilbert et al., 1998 with permission Virology).
1.6.1 Acute DHBV infection

Similar to HBV infection in humans, adult ducks are more capable of resolving high-dose DHBV infection compared with young ducks (Jilbert et al., 1998). In acute DHBV infection, DHBsAg is cleared from the serum by 1-2 months post-inoculation and is accompanied by lifelong anti-DHBc antibodies and anti-DHBs antibodies that protect against re-infection (Jilbert et al., 1998; Le Mire et al., 2005). Similar to residual HBV infection, residual DHBV DNA can be detected in the serum and liver of ducks that have resolved their DHBV infection. It has been shown that cccDNA is the major form of residual DHBV DNA found in the liver of ducks that have recovered from acute DHBV infection (Le Mire et al., 2005; Reaiche et al., 2010).

1.6.2 Persistent DHBV infection

In persistent DHBV infection, DHBsAg and anti-DHBc antibodies are readily detected in the serum. Anti-DHBs antibodies are usually undetectable in the serum but the circulating DHBsAg may mask their detection. In persistently DHBV-infected ducks, more than 95% of hepatocytes support DHBV replication and are DHBsAg-positive and high titres of DHBV DNA are found in the liver. Only mild or moderate inflammation is observed in the liver of persistently infected ducks with no reports of cirrhosis or HCC in experimentally DHBV-infected ducks (Foster et al., 2003; Le Mire et al., 2005; Reaiche et al., 2010). DHBV-infected ducks do not develop cirrhosis or HCC. The relative lack of exposure to alcohol and shorter life-span of ducks might play a role in this (Jilbert and Kotlarski 2000).

1.6.3 CMI responses to DHBV infection

CMI responses are important in both acute and persistent infection. It is accepted that the clearance of DHBV infection is through the elimination of DHBV-infected hepatocytes by CTL (Miller et al., 2008; Feng et al., 2010). There have been several studies on CMI
responses to DHBV and has provided (Jilbert and Kotlarski, 1999; Vickery et al., 1999; Welschinger et al., 2006).

In Vickery et al., 1999, the CMI responses to DHBsAg and DHBcAg have been measured using blastogenesis assay. To achieve this, duck peripheral blood (PBMC) and spleen mononuclear cell (SMC) were collected from uninfected control ducks, ducks acutely or chronically infected with DHBV and duck immune to DHBV. Cells were cultured and radioactive labelled for $^3$H thymidine. The PMBC and SMC responses to DHBsAg and DHBcAg were measured by $^3$H uptake.

In this study, acute DHBV infection was established by inoculation of nine 4-week-old ducks with DHBV using various pre-determined doses ($10^6$ID$_{50}$, $10^5$ID$_{50}$ and $10^5$ID$_{50}$). The results shows that three ducks failed to clear DHBV infection and had no response to DHBsAg and DHBcAg by PMBC. This indicates the DHBsAg and DHBcAg specific T-cells may play an important role in clearance of DHBV infection.

To establish chronic DHBV infection, three ducks were infected neonatally and two out of the three ducks developed chronic DHBV infection. In neonatal HBV infection, cellular infiltration of the liver was not observed until adulthood (Alexander, 1990). Similarly, neonatal infected ducks that developed chronic DHBV infection had an increasing CMI response over time. This low yet detectable T cell activity could be due immuno-tolerance in these ducks, in consistent with the observation in HBV infection (Rehermann et al., 1996).

Eight ducks were immunised following vaccination with purified DHBsAg on day 6, 12 and 26 post-hatch. These ducks were challenged with $5 \times 10^7$ ID$_{50}$ doses of DHBV 1 week prior to testing their cellular immune responses. Significant CMI responses were observed in these ducks following DHBV challenge, suggesting the involvement of CMI responses specific to DHBV antigens during the clearance of DHBV infection.
Therefore, this study has provided some experimental evidence for CMI responses in duck. This assay can be used to gain the better understanding of CMI responses during the course of DHBV infection.

1.7 Treatment of HBV infection

It is recognised that nuclear cccDNA serves as the template for viral transcription and is a key molecule that helps to maintain CHB infection. This constant virus replication in the hepatocytes leads to immune mediated hepatocyte damage and subsequent liver complications such as cirrhosis and HCC (Lai et al., 2010). Therefore, the ideal therapeutic result is the control of HBV infection and the eradication of the nuclear cccDNA. However, current therapies including immune-modulators and various antiviral agents including nucleot(s)ide analogues (NAs) are limited to suppressing virus replication to improve liver histology with normalization of ALT, or at least to delay the progression towards liver complications (Krastev, 2006).

1.7.1 Immunomodulators

Currently, two immunomodulators, the conventional IFN-α and pegylated (Peg) IFN-α2A, are approved for treatment of CHB. Both conventional and PegIFN-α2A are administered by injection and have antiviral and anti-proliferative effects. They promote specific T cell responses for lysis of infected hepatocytes and produce antiviral cytokines to control HBV replication (Karayiannis, 2003; Osborn and Lok, 2006).

The effectiveness of current therapies is normally assessed by a loss of serum HBV DNA, normalisation of ALT level, seroconversion from HBeAg to anti-HBe antibodies and HBsAg to anti-HBs antibodies. A long-term follow up of patients treated with IFN-α showed that IFN-α therapy resulted in sustained immune responses, improved histological conditions and
an increased HBeAg and HBsAg seroconversion rates (Van et al., 2004). Nonetheless, conventional IFN-α is only effective in approximately 30% of treated patients, especially those with raised ALT levels (Wong et al., 1993; Karayiannis, 2003; Van et al., 2004). In addition, conventional IFN-α therapy is less effective in Asian patients compared to Caucasian patients. This difference is thought to be due to early exposure to HBV leading to immunological tolerance in Asian patients (Lok et al., 1988; Shi et al., 2006).

PegIFN-α2A is created by attaching a 40-kDa polyethylene glycol polymer molecule to IFN-α2A to improve its pharmacokinetics. A recent study has shown that patients treated with PegIFN-α2A have a better treatment outcome compared with patients treated with conventional IFN-α in terms of rate of seroconversion from HBeAg to anti-HBe antibodies. This study also showed that PegIFN-α2A in combination with Lamivudine had no added efficacy over Lamivudine alone (Lau et al., 2005, Ferir et al., 2008).

The major advantages for IFN-α based therapy include higher rates of HBsAg loss, a more durable response and the absence of drug-resistance. However, IFN-based therapy is also limited by low HBeAg seroconversion rate, unpleasant side-effects and is mainly effective in groups of patients with high pre-treatment ALT levels and low viral load. In addition, IFN-α based therapy is ineffective in immunosuppressed patients and patients with decompensated cirrhosis. To treat immunosuppressed patients and decompensated cirrhosis patients, the only available option is an antiviral agent, e.g. nucleot(s)ide analogues (NA) (Karayiannis, 2003; Van et al., 2004; Krastev, 2006; Osborn and Lok, 2006; Lai et al., 2010).

1.7.2 Antiviral agents

Currently, the NA Lamivudine (3TC), Adefovir (ADV), Tenofovir (TFV) and Entecavir (ETV) have been approved in Australia to treat patients with CHB (Hepatitis Australia, 2010). All oral dosing NA treatments have been shown to reduce levels of HBV DNA and to exhibit
fewer side-effects compared to IFN-α based therapy. Furthermore, NAs are effective in all ethnic groups and against all HBV genotypes (Min and Dienstag, 2007). NAs inhibit the HBV Pol by competing with cellular dNTPs by high affinity binding to the HBV Pol, incorporation into the growing chain of DNA and subsequent DNA chain termination. This NA-induced DNA chain termination prevents the formation of RI DNA and new rcDNA as well as preventing the release of progeny virus to infect susceptible hepatocytes (Karayannis, 2003; Younger et al., 2004; Fabien, 2005; Osborn and Lok, 2006; Ferir et al., 2008). NA therapy by inhibiting HBV replication and the production of relaxed circular DNA also reduces levels of synthesis of new molecules of cccDNA.

Treatment with NAs has been shown to alter the outcome of CHB infection. In some treated patients, levels of HBV replication and antigen expression are reduced to a level that allows reactivation of an immune response to HBV infection. The change in the patients’ immune responses ultimately results in lower levels of virus replication, seroconversion from HBeAg to anti-HBe antibodies and improvement of liver histology but the patients remain positive for serum HBsAg (Xu and Chen, 2006; Min and Dienstag, 2007; Ferir et al., 2008; Chen et al., 2011).

1.7.2.1 Entecavir (ETV)

In this project, ETV has been used as part of the therapeutic strategy and is supplied by Bristol-Myers Squibb Pharmaceuticals. ETV is a NA and has been available for treatment of CHB infection since 2007. It has been shown that ETV is a potent antiviral drug to treat CHB virus infection with minimal side effects and only low rates of antiviral drug resistance (Min and Dienstag, 2007; Hepatitis Australia, 2010). Its active triphosphate form has a high affinity for the HBV polymerase and acts as a potent competitive inhibitor of natural substrate (dGTP) which results in DNA chain termination (Xu and Chen, 2006; Ferir et al., 2008).
Two double-blind Phase III clinical studies in HBeAg-positive and HBeAg-negative CHB patients showed that ETV treatment led to better therapeutic outcomes (e.g. reduction in HBV DNA levels and HBeAg loss), improved liver histology with reduced ALT levels and better efficacy compared to Lamivudine (Chang et al., 2006; Lai et al., 2006). ETV also showed potent antiviral activity in Lamivudine-resistant HBV (Suzuki et al., 2008). Another recent study showed that ETV had better efficacy in lowering HBV DNA levels in HBeAg-positive patients compared to Adefovir (ADV). However, no difference was observed between ETV- and ADV-treated HBeAg-negative patients (Chen et al., 2011). In addition, ETV demonstrated the ability to inhibit WHV replication in woodchucks (Colonno et al., 2001) and DHBV in ducks, resulting in decreased serum levels of viral antigen and DHBV DNA (Foster et al., 2003).

1.7.2.2 Antiviral resistance to Nucleotide/nucleoside analogues

One of the major disadvantages of NA treatment of CHB infection is it has little effect on existing cccDNA formed after viral entry. So far, none of the approved NAs target existing cccDNA or conversion of rcDNA into cccDNA in the nucleus of infected hepatocytes after initial infection. This indicates viral eradication and elimination of cccDNA are not able to be achieved and thus impede the clearance of CHB infection. This is because residual viraemia may still be able to cause infection of susceptible hepatocytes during antiviral treatment (Zoulim, 2005; Kwon and Lok, 2011).

In addition, since viral eradication and elimination of cccDNA are not able to be achieved, long-term administration of NA treatment is needed. This is because in most of the CHB patients, immune response against HBV is defective and thus withdrawal of the antiviral treatment results in rebound of the virus replication (Zoulim, 2005; Xu and Chen, 2006). Therefore long-term administration of NA should be considered since sustained virological
responses are infrequent and rebound of virus replication is common in CHB patients (Xu and Chen, 2006; Kwon and Lok, 2011).

Also, the major concern of this prolonged NA treatment is the emergence of NA-resistant HBV mutant strains, generated by the spontaneous error rate of the viral Pol (Osborn and Lok 2006; Lai and Liao 2010). Under the pressure of an antiviral drug, HBV strains harbouring a mutation that confers resistance to the NA are selected. For instance, this mutation could alter the structure of the HBV Pol and decrease its affinity with the NA (Papatheodoridis and Dimou, 2002; Karayiannis, 2003; Wang et al., 2009). This NA-resistant HBV mutant strain is then likely to become dominant over the wild-type HBV strain due to the growth advantage of the NA-resistant-strain. Furthermore, resistance to one NA may reduce future treatment options as cross-resistance to other agents is possible (Krastev, 2006; Min and Dienstag, 2007; Kwon and Lok, 2011).

Therefore, developing and improving novel immune therapies and enhancing existing therapies by using appropriate molecular adjuvants that can stimulate host immune response to sustain virus suppression and CMI response to target infected hepatocytes before the emergence of drug-resistant HBV strain may lead to better treatment outcomes for CHB.

1.8 Hepadnavirus vaccines

1.8.1 The prophylactic HBV vaccines
Currently, two types of prophylactic HBV vaccines are available. The first type of HBV vaccine, also the prototype vaccine, was prepared by purifying 22 nm sub-viral HBsAg particles directly from the serum of chronic HBV carriers. This prototype vaccine which is inactivated and combined with aluminium hydroxide induces high-titres of neutralising anti-
HBs antibodies that recognise the “a” determinant, a common epitope within the HBsAg of all HBV subtypes (Szmuness, 1979).

The second type of HBV vaccine, which is a recombinant HBsAg subunit vaccine, is produced through expression of plasmid DNA encoding HBsAg in yeast. These recombinant HBsAg (rHBsAg) particles are obtained and purified by lysing of the yeast cells to release the rHBsAg particles which are combined with aluminium hydroxide to form the HBV vaccine. Since the rHBsAg particles do not contain the viral DNA but only HBV surface structure, they do not carry any risk of HBV infection. Yeast-derived HBV vaccines are therefore safe and have been widely used. Administration of three intramuscular (i.m.) doses of HBV vaccine over ~6 months usually induces a strong anti-HBs antibody response that confers protection against HBV infection in healthy infants, children, and young adults (Da Villa et al., 1998; Mast et al., 2005; WHO, 2011). However, these HBV vaccines fail to induce anti-HBs antibodies and failed to confer protection against HBV infection in 5-10% of recipients (Mast et al., 2005). Currently, infants born to HBV carrier mothers are vaccinated with the HBV vaccine and injected with HBV immunoglobulin soon after birth. This regimen is effective in preventing vertically transmitted HBV infection in >90% of newborn infants (Mast et al., 2005).

These two types of prophylactic vaccines, while inducing strong protective immunity, provide no therapeutic benefit to patients with CHB as only a strong humoral immune response is induced, without induction of a strong CMI (Fabrizi et al., 2004).

### 1.8.2 DNA vaccines

Several novel vaccines, e.g. DNA vaccines, have been tested against hepadnaviral infection models with the ultimate aim of developing novel therapeutic strategies for CHB or at least to delay the progression of cirrhosis and development of HCC (Mancini-Bourgine et al., 2004).
DNA vaccines are produced by cloning of the gene of interest downstream from a high-level expression promoter that operates in mammalian species. DNA vaccines deliver the gene of interest, which codes for a specific antigen, directly into cells of the immunised host. After inoculation, DNA vaccines are taken up by wide range of cell types such as myeloid cells, Langerhans cells and other APCs (Belakova et al., 2007). These DNA vaccine-coded antigens are then expressed intracellularly in a similar way to antigens during natural viral infection, without the risk of administration of infectious agents (Garmory et al., 2005).

Unmethylated cytosine-phosphate-guanosine (CpG) motifs are common in plasmid DNA (Gurunathan et al., 2000). CpG motifs are an important immunomodulatory component of DNA vaccines as they exert adjuvant effects on DNA vaccines to stimulate a Th1 response. These CpG motifs are recognised by pattern recognition receptors (PRR) present on APCs, e.g. TLR-9. The recognition of CpG motifs by APCs leads to the secretion of the Th1 type cytokines, e.g. interleukin-12 (IL-12), IFN-α, and interferon-beta (IFN-β) which are involved in the generation of CMI (Davis, 2000; Garmony et al., 2003). Interaction between CpG motifs and TLR-9 also results in the increased expression of co-stimulatory molecules on the APC, e.g. B7 molecules, adhesion molecule ICAM-1 and the CD40 receptor (Thalhamer et al., 2001). The recognition of CpG motifs also leads to the activation of intracellular signalling cascades in B lymphocytes that stimulate B lymphocyte proliferation and antibody production (Davis, 2000; Garmony et al., 2003).

DNA vaccination offers several major advantages over traditional vaccines, e.g. stimulation of specific humoral and CMI responses against cloned antigens; genes of several antigens can be incorporated into the same DNA vaccine and delivered to a host in a single dose; specificity of the DNA vaccine-encoded antigens expressed in host cells can closely resemble those of the natural infection; relatively cost-effective production has the potential for global use; and stability and easy storage of DNA (Liu, 2003). All of these characteristics have made
DNA vaccines a suitable candidate for prophylactic vaccination or immunotherapy for chronic viral infections, including HBV, hepatitis C virus (HCV), human immunodeficiency virus (HIV) and parasitic infections, such as malaria (Gurunathan et al., 2000; Beckebaum et al., 2002).

1.8.2.1 Mechanism of antigen presentation following DNA vaccination

Following DNA vaccination professional APCs, (e.g. dendritic cells (DCs), macrophages and B lymphocytes) can present the antigens in the context of both MHC class I and MHC class II molecules and thus play a central role in the regulation of both T and B cell responses to DNA vaccines. In general, DCs are more potent at initiating immune responses to the DNA vaccine encoded antigens than other professional APCs. This is due to the expression of co-stimulatory molecules, e.g. B7 molecules on the surface of DCs that are required for the priming of naïve T lymphocytes (Gurunathan et al., 2000; Belakova et al., 2007).

Although immunisation with DNA vaccines has been proved to induce specific CMI and humoral responses, the exact mechanism is not fully understood. Immunisation with DNA vaccines stimulates the activation of CD4+ and CD8+ T lymphocytes through both endogenous (MHC class I-restricted) and exogenous (MHC class II-restricted) antigen presentation pathways. After DNA vaccination, three possible mechanisms have been suggested to explain induction of a MHC class I-restricted CTL response: a) expression of peptides of cloned antigen in the context of MHC class I complex at the surface of any transfected somatic cells (e.g. myocytes, keratinocytes, or any MHC class II-negative cells); b) direct transfection of any professional APCs and with antigen presentation through the MHC class I pathway; c) cross-priming of APCs when DNA vaccine-transfected somatic cells are engulfed by APCs and the DNA vaccine or expressed antigen is subsequently presented by the MHC class I pathway to CD8+ T lymphocytes. This cross-priming phenomenon is defined as the re-presentation of exogenously derived cell-associated antigens on MHC
molecules for priming of CD8+ T lymphocytes (Belakova et al., 2007; Rajcani et al., 2005) (Figure 1.7).

On the other hand, DNA vaccine-coded antigens that are secreted from the transfected cells can be processed as exogenous antigens and presented by the MHC class II pathway. These result in a preference for generating MHC class II-restricted Th2 cell responses and specific humoral responses against cloned antigens (Liu, 2003; Belakova et al., 2007).

1.8.2.2 Enhancement of the immunogenicity of DNA vaccines

The major limitation of DNA vaccination is that DNA vaccines are not highly immunogenic, and has been found to be inefficient at generating immune responses in clinical trials in humans (Mancini-Bourgine et al., 2004; Mancini-Bourgine et al., 2006), in large animals (Babiuk et al., 1999) and non-human primates (Kim et al., 2000).

In recent studies from our laboratory, DHBV DNA vaccines used in combination with the antiviral drug ETV for treatment of persistent DHBV infection, did not lead to sustained responses (Foster et al., 2003), similar to the findings of a HBV DNA vaccine study in chronically HBV-infected humans (Mancini-Bourgine et al., 2004). This has indicated that DNA vaccines when used alone, or in combination with antiviral drug, e.g. ETV, may not be immunogenic enough for use as a therapeutic HBV vaccine.

To further optimise DHBV DNA vaccination, two strategies have been used in this thesis to enhance immune responses: (i) the use of a genetic adjuvant e.g. a DNA vaccine expressing CD40L, and (ii) the use of heterogeneous prime-boost vaccination strategy utilising DHBV DNA vaccines to “prime” and recombinant poxvirus vaccines to “boost” the immune response. These studies will be discussed in Section 1.10.1.
1.8.2.3 Co-stimulatory molecules as genetic adjuvants

To further increase the efficiency of DNA vaccines, powerful genetic adjuvants that can safely enhance the specific T lymphocyte immune responses without severe side-effects are needed. The full activation of T lymphocytes requires two essential signals from APC. The first signal is antigen specific and is delivered through the T cell receptor (TCR) which interacts with the antigen-MHC complex on the APC. The second signal, also known as a co-stimulatory signal, is antigen non-specific and is mediated through the interaction between co-stimulatory molecules expressed on the T lymphocyte and the APC (Ahlers et al., 2003; Duttagupta et al., 2009).

Many studies have demonstrated that the interaction of co-stimulatory molecules found on T lymphocytes and APC is critical for the full activation of T lymphocytes (Iwasaki et al., 1995; Girvin et al., 2000; Scandiuzzi et al., 2011). The reciprocal interaction between B7 molecules on DC and CD28 on T lymphocytes is well studied and is the best example to illustrate the importance of co-stimulatory molecules. After the first signal is delivered through the TCR, interaction between the B7 molecule on DC and CD28 on T lymphocytes leads to the full activation of T lymphocytes. In addition, the interaction between B7 and CD28 molecules up-regulates CD40L on T lymphocytes which then interact with CD40 on APC to further increase the expression of B7 on the APC. Activated T lymphocytes secrete numerous cytokines, e.g. IL-2 that can enhance proliferation and differentiation of T lymphocytes. In contrast, without the interaction between B7 and CD28 molecules, T lymphocytes can never be activated and will end up in anergy or the apoptosis pathway (Ahlers et al., 2003). In order to enhance vaccine-induced immune responses to HBV antigens, here we explore the role of another co-stimulatory molecule, CD40L, a potential component of DNA vaccines.
**Figure 1.7:** Mechanism of antigen presentation following DNA vaccination for induction of a MHC class I-restricted CTL response.

Three possible pathways have been suggested:

a) Indirect route: expression of processed antigen in the context of MHC class I complex at the surface of any transfected somatic cells (e.g. myocytes, keratinocytes, or any MHC class II-negative cells).

b) Direct route: direct transfection of any professional APCs with encoded antigen processed endogenously and presented through the MHC class I presentation pathway.

c) Cross-priming of APC: DNA vaccine-transfected somatic cells are engulfed by APCs and subsequently presented via MHC class I to CD8+ T lymphocytes.

Obtained from Rice *et al.*, 2008 with permission from Nature Reviews Cancer.
1.8.2.4 The co-stimulatory molecule CD40L

CD40L, also known as CD154, is a type II integral membrane glycoprotein and a member of the TNF-α family of cell surface interaction molecules (Daoussis et al., 2004). The crystal structure of CD40L consists of an intracellular N-terminal domain, a short trans-membrane segment, a relatively long extracellular ‘stalk’ and a globular TNF-like extracellular domain at the C-terminal end. The globular regions are predicted to be involved in CD40 binding (Karpusas et al., 1995). CD40L is mainly expressed on the surface of activated CD4+ T lymphocytes, but it can also be found on many cells of the immune system, such as B lymphocytes, NK cells and endothelial cells. On the other hand, its receptor, CD40, is constitutively expressed mainly on APC, including B lymphocytes, macrophages and DCs (Blossom et al., 1999; Higuchi et al., 2002; Jyothi et al., 2000; Mach et al., 1997).

In general, CD40L plays the role of a co-stimulatory molecule and the direct consequence of the CD40L-CD40 interaction is dependent on the partner cell type (Figure 1.8). Engagement of CD40L with CD40 on B lymphocytes results in increased expression of B7 on B lymphocytes, which subsequently promotes better interaction with CD28 on T lymphocytes. Consequences of this activation cascade include B lymphocyte differentiation, B lymphocyte proliferation and immunoglobulin (Ig) isotype switching (Garside et al., 1998; Aruffo et al., 1993).

The interaction of CD40L with CD40 expressed on DC induces activation of DC, DC migration to lymph nodes, and can prolong the survival of activated DC. This improves the ability of DC to prime CD8+ T lymphocytes, leading to production of inflammatory cytokines and chemokines that enhance antigen-specific immune responses (Daoussis et al., 2004; Fraser et al., 2007).
CD40L on CD4+ T lymphocytes has been shown to license DC via CD40 to prime CTL responses (Bennett et al., 1998; Schoenberger et al., 1998; Smith et al., 2004). This process is to stimulate production of memory CTLs and is known as cross-priming (Borrow et al., 1996; Sun et al., 2003).

Interaction of CD40L with CD40 expressed on macrophages increases surface expression of CD40 and the TNF receptor, which enhances the level of macrophage activation. Activated macrophages can then destroy phagocytosed foreign materials and produce more cytokines.

Therefore, the CD40–CD40L interaction plays a central role in development of both humoral and CMI.

The recognised importance of the CD40L–CD40 duet during immune responses has led to extensive studies using these molecules as therapies by either inhibiting or activating the interaction. Monoclonal antibodies (MAb) against CD40L have been used as an immunosuppressive treatment in many animal transplantation models (Kirk et al., 1999; Kvist et al., 2008; Mungara et al., 2008). In contrast, there is an increasing focus on the use of CD40L as an immuno-modulator for DNA vaccination in anti-tumour and anti-viral therapies (Huang et al., 2004; Peter et al., 2002; Stone et al., 2006).

Notably, expression of CD40L is transient and the rapid disappearance from the surface of activated T lymphocytes is observed after the initiation of lymphocyte activation in humans (Fuleihan et al., 1994). This implies that only a short period of opportunity is given for activated CD4+ T lymphocytes to deliver helper signals to APC (Fuleihan et al., 1994; Daoussis et al., 2004; Fraser et al., 2007). Hence, a CD40L expressing DNA vaccine may help to generate effective humoral and CMI against virus infections.
**Figure 1.8:** Cognate interaction between CD40 and CD40L and their impact on CMI and humoral immune responses.

Engagement of **CD40L** with **CD40 on B lymphocytes** results in increased expression of B7 on B lymphocytes, which subsequently promotes better interaction with CD28 on T lymphocytes. Consequences of this activation cascade include B lymphocyte differentiation, B lymphocyte proliferation and immunoglobulin (Ig) isotype switching (Garside *et al.*, 1998; Aruffo *et al.*, 1993).

The interaction of CD40L with CD40 expressed on APC induces activation of APC. Subsequently, CD40L on CD4+ T cells has been shown to license APC via CD40 to prime CTL responses (Bennett *et al.*, 1998; Schoenberger *et al.*, 1998; Smith *et al.*, 2004) and stimulate production of memory CTLs (Borrow *et al.*, 1996; Sun *et al.*, 2003). This process is known as cross-priming.
MHC-I

CD8+

peptide

Activated APC

TCR

CD8

B lymphocyte

peptide

Th2

Plasma cell

Antibodies

Th1

Activated APC

MHCI

peptide

CD8+

CD4+

CD4

CD40L

CD28

B7

CD4

CD40

CD40L
1.8.2.5 Current Hepadnavirus vaccine studies

As mentioned above, the current HBsAg vaccines provide no therapeutic benefit to patients with CHB. DNA vaccines have been tested against HBV infection with the ultimate aim of developing novel therapeutic strategies for CHB carriers or at least to delay the progression of cirrhosis and HCC development. Numerous studies have demonstrated the ability of DNA vaccines to induce specific humoral and CMI responses against HBV in humans and transgenic mice, WHV in woodchucks, and DHBV in ducks (Lu et al., 1999; Mancini-Bourgine et al., 2004; Mancini-Bourgine et al., 2006; Rollier et al., 1999; Triyatni et al., 1998; Foster et al., 2005; Xing et al., 2005). A protective vaccine study conducted in our laboratory has shown that DHBV DNA vaccines expressing the DHBV S and pre-S/S genes generated protective immunity in young and adult ducks (Triyatni et al., 1998). Another study showed that vaccination of ducks with novel whole-cell vaccines derived from primary duck embryonic fibroblasts (PDEF) transfected with DNA vaccines expressing DHBV core antigen, resulted in protection against the development of persistent DHBV infection (Miller et al., 2006a). Recently, we also reported the development of a successful post-exposure therapy that combined treatment with the NA ETV, and prime-boost DNA vaccines expressing DHBV surface and core antigens, and prevented the development of persistent DHBV infection (Miller et al., 2008, Feng et al., 2010).

According to a US database at http://clinicaltrials.gov as of March 2014 if we refine the search to plasmid (naked) DNA vaccines, there are 64 registered clinical trials (regardless of their trial Phase) and four of them are HBV DNA vaccine related. In one of the completed HBV DNA vaccine related Phase I clinical trials, the HBV-specific T lymphocyte responses were assessed by cell proliferation, ELISpot and cell tetramer assays in response to HBV viral proteins. In the study, only weak and transient restoration of T lymphocyte mediated immune responses were observed in CHB carriers after DNA vaccination and this response was not strong enough to control HBV infection (Mancini-Bourgine et al., 2004). The poor immune
response after DNA vaccination in many clinical trials has become a challenge for future DNA vaccine development.

1.9 Poxviruses

1.9.1 Family Poxviridae

The poxvirus family is divided into two sub-families: Entomopoxviridae and Chordopoxvirinae. The sub-family Entomopoxviridae is classified based on their invertebrate host range and infect insects. The family Chordopoxvirinae infects vertebrate hosts and is subdivided into eight genera: Parapoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Yatapoxvirus, Molluscipoxvirus, Orthopoxvirus and Avipoxvirus (Beukema et al., 2006). Half of these genera (Parapoxvirus, Yatapoxvirus, Molluscipoxvirus, Orthopoxvirus) can infect humans, e.g. the Orthopoxvirus variola (or small pox) and the Molluscipoxvirus Molluscum contagiosum. Members of the Chordopoxvirinae sub-family share several common features: all are large double-stranded DNA viruses with genomes that vary in size, from 130-365 kb, and they replicate in the cell cytoplasm (McFadden 2005; Weli and Tryland, 2011).

Avipoxvirus (APV) is the only genus in the Chordopoxvirinae sub-family that can infect non-mammalian hosts and APV commonly infect avian species (Beukema et al., 2006). This genus is transmitted by aerosols and biting insects, causing diseases in poultry, pets and wild birds. Currently, full genome sequences are only available for Canarypox virus and Fowlpox virus (FPV) in this genus, and FPV is the best studied member of the Avipoxviruses (Weli and Tryland, 2011).

1.9.2 Structure and genome of Avipoxviruses

Avipoxviruses share several biological features with other poxviruses. All are large enveloped viruses with two lateral bodies and a centrally located viral nucleocapsid. Avipoxviruses are double-stranded DNA viruses, with genomes ranging in size from 260 to 365 kbp that contain
several hundred closely spaced ORF. The central region of avipoxvirus genomes includes two identical inverted terminal repeats (ITRs) that are linked by hairpin loops. Many of the APV ORF is conserved among the various poxviruses and at least 90 of them are required for steps in poxvirus replication, e.g. viral transcription, viral DNA replication and virion assembly (Mcfadden, 2005; Weli and Tryland, 2011).

1.9.3 Replication of Avipoxviruses

The replication cycle of Avipoxviruses is a complicated cytoplasmic event due to the existence of two types of distinct infectious virus particles, namely the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). The IMV and EEV differ in surface glycoprotein and lipid membranes. APV bind to an unknown receptor on host cell membranes. Fusion between the virus and the host cell membrane results in the release of the virus nucleocapsid into the cytoplasm of the host cell (Mcfadden, 2005; Weli and Tryland, 2011).

APV replication is characterised by three stages of viral mRNA and protein synthesis which are under the control of early, intermediate and late gene promoters. At the first stage, endogenous RNA polymerase and transcription factors from the virion core start synthesizing viral mRNA under the control of I early gene promoters. The second stage is the poorly understood core un-coating process that results in the release of viral DNA into the cytoplasm of the host cell, to either serve as a template for viral DNA replication or for transcription of intermediate and late viral gene mRNA. Lastly, the accumulation of the late or structural gene products enables assembly and maturation of infectious virus particles. Typically, replication of these viruses in the cytoplasm of infected avian cells results in a cytopathic effect (CPE) within 4 to 6 days p.i. (Mcfadden, 2005; Weli and Tryland, 2011).
1.9.4 FPV as vaccine vectors

Effective vaccines should generate optimal humoral and CMI responses. However, in general, inactivated or subunit vaccines induce humoral but not CMI responses that are required to combat many infectious diseases and cancers. Recombinant APV-based vectors appear to be ideal candidates for the development of vaccines that induce optimal humoral and CMI responses.

FPV, which primarily infects chickens and turkeys, is a large enveloped virus containing a linear double-stranded DNA of ~300 kbp in size (Afonso et al., 2000). FPV is unable to fully replicate in mammalian cells and this helps to make FPV a safe vaccine vector in humans (Diener et al., 2008). The large genome of FPV allows the insertion of multiple genes, including viral antigens, cytokines and co-stimulatory molecules that may restore the immune response and potentially control disease progression (Zanotto et al., 2010). For instance, recombinant FPV (rFPV) strains, containing multiple inserted genes, can infect mammalian cells to allow expression of cloned antigens under the control of early gene promoter. This in turn can lead to the induction of specific immune responses against cloned vaccine antigens (Lousberg et al., 2011). The exact mechanism of FPV interaction with the mammalian immune responses is still poorly understood. However, it is believed that the intracellular expression of cloned viral antigens in local APCs in the context of MHC class I and/or MHC class II molecules leads to the activation of specific CD4+ and CD8+ T lymphocytes against cloned antigens (Diener et al., 2008). Currently, several FPV-vectored vaccines have been licensed for commercial use in chickens as vaccines against avian influenza virus (Bublot et al., 2006), Newcastle disease virus (Sun et al., 2008), infectious laryngotracheitis virus (Davison et al., 2006), and avian encephalomyelitis in North America (Zhang et al., 2010).

Recombinant FPV vector based vaccines have been used in many experimental studies against various infectious diseases in animals (Hsu et al., 2010; Johnson et al., 2010), and
promising results have been obtained particularly when used as part of prime-boost vaccination regimens in human diseases (Madan et al., 2007; Kraufman et al., 2007; Greenough et al., 2008; Lechleider et al., 2008; Fraser et al., 2010; Radaelli et al., 2010). Despite increasing interest in rFPV as prophylactic or therapeutic vaccines for various human diseases, only a few FPV vector based HIV, malaria and cancer vaccines have been tested in clinical trials, and most of them are used as part of prime-boost vaccination regimens (Pialoux et al., 2001; Imoukhuede et al., 2006; Kaufman et al., 2008; Rerks-Ngarm et al., 2009). In one of the recent phase I HIV clinical trials, two different poxvirus vectors (modified vaccinia Ankara [MVA] and FPV) expressing the same HIV insert, namely MVA-HIV and FPV-HIV, in a heterologous prime-boost vaccination regimen were tested as preventive HIV vaccines. The results showed that both MVA-HIV and FPV-HIV vaccines were well tolerated in all participants. Furthermore, significant HIV-specific CD4+ and CD8+ T-cell responses were induced in ~50% of the recipients when MVA-HIV was used to prime twice, followed by FPV-HIV to boost under the prime-boost vaccination regimen (Keefer et al., 2011). Although these promising results have not yet been tested on HIV-infected patients, it has shown the potential of a FPV vector based vaccine in improving the quality and strength of the vaccine-induced immune response in humans. As a note of caution, lack of information on how FPV vector based vaccines interacts with the mammalian immune system may undermine the effort for vaccine improvement (Beukema et al., 2006).

1.9.5 Poxvirus vaccines developed to treat hepadnavirus infection

FPV-M3 is a commercially available attenuated avian vaccine strain of FPV used in chickens to protect against wild-type FPV. It is produced by Fort Dodge Pty. Ltd. and has been approved for use as a vaccine vector in chickens, mice, macaques and humans (Boyle et al., 1997).
In this current study, rFPV strains expressing DHBV antigens (rFPV-DHBV) were derived from FPV-M3. The rFPV-DHBV strains expressing DHBV core and pre-surface antigens (rFPV-DHBc and rFPV-pre-S/S) were constructed in collaboration with David Boyle, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Geelong, Australia (Miller et al., 2008).

The rFPV-DHBV strains were constructed by cloning the DHBV core and pre-surface genes into a “shuttle vector”, pAF09 that allows insertion of foreign genes into the non-essential thymidine kinase gene of FPV-M3, downstream of the FPV early gene promoter (Miller et al., 2008). Chicken cells infected with FPV-M3 were transfected with the pAF09-DHBV constructs allowing homologous recombination as previously described in Miller et al., 2008. Western blot analysis of primary chicken embryonic fibroblasts (PCEF) and primary duck embryonic fibroblasts (PDEF) infected with these rFPV-DHBV strains confirmed the expression of the inserted genes (Miller et al., 2008; Feng et al., 2010).

In a recent post-exposure study, ETV treatment was combined with heterologous “prime-boost” vaccination that utilises sequential immunisation with DNA vaccine to “prime” followed by rFPV-DHBV to “boost” the specific immune responses (Miller et al., 2008). In Chapter 6, studies combining ETV treatment with “prime-boost” vaccination were extended to determine the essential components of this vaccination strategy.

1.10 Research aims and study outline

The ultimate aim of this Ph.D. project was to develop and assess therapeutic vaccination strategies that induce immune responses that target virus infected hepatocytes and allow successful control of chronic HBV infection. The studies were preformed in DHBV-infected ducks, used a numbers of assays such as ELISA, qPCR, immuno-staining of DHBV antigens to determine the induced immune responses in ducks following vaccination.
As the first aim, rDHBcAg was purified and used to immunise rabbits and mice to produce specific polyclonal and monoclonal antibodies for the detection of DHBcAg. The polyclonal and monoclonal anti-core antibodies were then used to optimise Western Blot, immunofluorescent and immuno-staining of DHBV core antigen in fixed liver tissues to assess the efficacy of the different vaccination strategies throughout this study. The sensitivity and specificity of immuno-staining detection of DHBcAg in duck liver sections was compared to the detection of DHBsAg to allow comparison of the 2 techniques for the detection of DHBV-infected hepatocytes. These studies are described in Chapter 3 of this thesis.

To further improve the immune response induced by DHBV DNA vaccines, the DuCD40L cDNA sequence was cloned using mRNA from duck PBMC into a DNA vaccine vector and was subsequently tested for bioactivity using in vitro assays. The cloning and analysis of the CD40L was performed in our laboratory by Dr Feng Feng. Subsequently, as reported in Chapter 4 of this thesis the DNA vaccine expressing DuCD40L was incorporated into a study in 6-week-old ducks to assess the protective efficacy of DHBV DNA vaccines with and without co-administration of the DuCD40L DNA construct.

Chapter 5 aimed to further confirm the protective efficacy of DHBV DNA vaccines co-administered with a DuCD40L DNA construct, against DHBV infection in 14-day-old ducks which are more susceptible to the development of persistent DHBV infection.

ETV treatment combined with heterologous “prime-boost” vaccination expressing the DHBV core and surface antigens has been previously shown to prevent the development of persistent DHBV infection when administered as a post exposure therapy (Miller et al., 2008; Feng et al., 2010). In Chapter 6 of this thesis, these studies were extended to determine if the DHBV core or surface antigens, were capable of inducing efficient humoral
and CMI in DHBV-infected ducks, and eventually prevent the development of persistent DHBV infection. Finally, a broad conclusion of the work performed for this thesis and a discussion of future work are presented in Chapter 7.
Chapter 2: Materials and Methods

2.1 Experimental animals

1-day-old DHBV-negative Pekin-Aylesbury ducklings (*Anas domesticus platyrhynchos*) were obtained from a commercial poultry hatchery (Pepe’s ducks, New South Wales, Australia) and housed and maintained in the SA Pathology Veterinary Division Animal Care Facilities at Frome Road, Adelaide or at Gilles Plains, South Australia.

The mice used for production of monoclonal antibodies (mABs) were ~6-week-old specific-pathogen-free BALB/c laboratory mice (*Mus musculus*). The rabbits used for production of polyclonal antibodies were 12-week-old pathogen-free white rabbits (*Oryctolagus cuniculus*). All experimental mice and rabbits were obtained from and housed at the SA Pathology Animal Care Facilities at Gilles Plains, South Australia.

All animal experimentation and animal handling procedures were reviewed and approved by the Animal Ethics Committees of the Central Northern Adelaide Health Service and The University of Adelaide and followed the guidelines of the National Health and Medical Research Council (NHMRC) of Australia.

2.2 Cell culture

2.2.1 Preparation of primary duck embryonic fibroblasts (PDEF)

PDEF were prepared using 12-day-old embryonated duck eggs. Embryos were removed from the eggs and were placed in a 10 cm plastic petri dish with Hanks’ balanced salt solution (HBSS, Cat # 14025-092, Invitrogen). The head, wings and the feet of each embryo were removed using a sterile scalpel blade. The embryos were then eviscerated aseptically using sterile scissors and forceps in the same dish. The remaining parts of the embryos were
dissected with scissors and digested in 0.1% (W/V) trypsin-EDTA (Cat. # 27250-18, Gibco) in phosphate buffered saline (PBS) pH 7.2 for 10 min at 37°C. After centrifugation at 1800 g for 2 min, the supernatant was removed and 20 mL of supplemented Dulbecco’s Modified Eagle’s Minimal medium (DMEM; Cat. # 11965-092, Gibco) (Table 2-1) was added to resuspend the cells. PDEF were then divided into 1x175 cm² tissue culture flask per embryo and were cultured in a 37°C incubator with 5% CO₂.

2.2.2 Collection and purification of duck peripheral blood mononuclear cells (PBMC)

Blood was collected from the jugular vein of DHBV-negative ducks and was added to an equal volume of heparinized PBS. An equal volume of this diluted blood was layered over lymphoprep (Cat. # 1114544, Fresenius Kabi Norge As, Norway) followed by centrifugation at 600g for 20 min at room temperature (RT). PBMC above the red cell pellet were collected and washed three times in HBSS, before use. The PBMC were cultured in Costar® 3524 24-well-plates (Corning Inc., USA) (8x10⁵ cells/well; Table 2-1) in a 37°C incubator with 5% CO₂.

2.2.3 Cell culture and PHA and ConA stimulation of spleen cells

Spleen tissue was collected from DHBV-negative ducks and cut into small sections before being homogenised with a tissue homogeniser (Cat. # 4-3398A, Aminco) in HBSS and filtered through cotton wool columns under sterile conditions. An equal volume of Ficoll-HyPaque was layered over the homogenate followed by centrifugation at 200 g for 20 min at 4°C. Cells at the interface were collected and centrifuged at 1800 g for 10 min. Pellets were washed in HBSS and centrifuged at 1800 g for 10 min, three times, before the pellets were resuspended in GI 1640 (Cat. # A10491-01 Gibco) cell culture medium as described in Table 2-1. Spleen cells were cultured in Costar® 3524 24-well-plates (8x10⁶ cells/well), in GI either
Table 2-1: The complete composition of media used for cell culture

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Base medium</th>
<th>Supplemented with</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDEF, HD11, 293T</td>
<td>DMEM (Cat. # 11965-092, Gibco)</td>
<td>10 mM hepes, 1.2 mg/L penicillin, 16 mg/L gentamycin, 1.5 g/L L-glutamine, 10% FCS</td>
</tr>
<tr>
<td>LMH, duck PBMC</td>
<td>GI 1640 (Cat. # A10491-01 Gibco)</td>
<td>1.2 mg/L penicillin, 16 mg/L gentamycin, 1.5 g/L L-glutamine, 10% FCS</td>
</tr>
<tr>
<td>dstet5 (repressed)</td>
<td>GI 1640</td>
<td>1.2 mg/L penicillin, 16 mg/L gentamycin, 1.5 g/L L-glutamine, 5% FCS, 200 μg/mL G418, 1 μg/mL tetracycline (Cat. # T7660-5G, Sigma-Aldridge)</td>
</tr>
<tr>
<td>dstet5 (induced)</td>
<td>GI 1640</td>
<td>1.2 mg/L penicillin, 16 mg/L gentamycin, 1.5 g/L L-glutamine, 5% FCS, 200 μg/mL G418</td>
</tr>
<tr>
<td>SP2/0</td>
<td>DMEM</td>
<td>10% FCS, 1.2 mg/L penicillin, 16 mg/L gentamycin</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>GI 1640 (Cat. # A10491-01 Gibco)</td>
<td>1.2 mg/L penicillin, 16 mg/L gentamycin, 1.5 g/L L-glutamine, 10% FCS treated with either PHA (5 μg/mL) or ConA (5 μg/mL)</td>
</tr>
</tbody>
</table>
untreated or stimulated with 5 \( \mu g/mL \) of phytohemagglutinin M (PHA; Cat. # L-8902, Sigma) or 5 \( \mu g/mL \) of concanavalin (ConA; Cat. # 17-0450-01, Pharmacia; Pfizer), in a 37°C incubator with 5% CO\(_2\).

### 2.2.4 Culture of HD11, 293T, LMH and dstet5 cells

Before culturing cells, tissue culture plates were coated with 0.1% gelatin in PBS at 37°C incubator for 30 min and gelatin was removed by aspiration. The chicken macrophage cell line (HD11) (Lowenthal et al., 1995), and 293T cells were cultured in supplemented DMEM medium (Table 2-1). The chicken hepatoma cell line LMH (Kawaguchi et al., 1987) (Cat. # CRL-2117, ATCC) and the LMH sub-line dstet5 (Guo et al., 2003) were cultured in G1 medium 1640 supplemented with phenol red (Cat. # A10941-01, Gibco). For each 6-well-plate, \( \sim 2 \times 10^6 \) cells were seeded per well whereas in each 24-well-plate, \( \sim 3 \times 10^5 \) cells were seeded per well. All cells were cultured in a 37°C incubator supplied with 5% CO\(_2\). Culture medium was replaced every 2-3 days or when it turned yellow in color. The complete composition of culture media for 293T, PDEF, LMH and dstet5 cells (repressed or induced) used in this project is listed in Table 2-1.

### 2.2.5 Cell transfection

When 293T and LMH cells were grown to 50-60% confluence, they were then transfected with plasmid DNA using FuGENE6® (Cat. # 11815091001, Roche). For each well of a 6-well-plate, 3 \( \mu L \) of FuGENE6 was added into 95.5 \( \mu L \) of serum-free DMEM medium followed by the addition of 1.5 \( \mu L \) of plasmid DNA at a concentration of 1 \( \mu g/\mu L \). The mixture was incubated at RT for 20 min and was added evenly into each well of 6-well-plate. The cells were incubated with transfection mixture for a minimum of 2 days before fresh medium was added. For each well of a 24-well-plate, 3 \( \mu L \) of FuGENE6, 46.2 \( \mu L \) of serum-free DMEM medium and 0.8 \( \mu g \) of plasmid DNA were added in a similar manner.
PDEF were transfected by electroporation using a Gene Pulser® apparatus (Cat. # 1256078, Bio-Rad). PDEF were grown to 70-80% confluent, trypsinized and subsequently resuspended in serum-free DMEM media. PDEF were then spun at 1800 g, 4°C, for 5 min and the cell pellet was resuspended in PBS at a concentration of 1x10^6 cells/mL. 30 µg of plasmid DNA with 5x10^6 PDEF cells were added together into an electroporation cuvette (Cat. # 1652081, Bio-Rad) and kept on ice. Electroporation of the PDEF with the Gene Pulser® apparatus was performed at a voltage of 0.35 kV, capacitance of 960 µFD, for ~3 sec. After electroporation, 1.5 mL of DMEM was added into each cuvette and the cell suspension was transferred to 6-well-plate and grown for 1-2 days until they were confluent.

2.2.6 Detection of DHBV antigens using immune-fluorescence (IMF) assay

Dstet5 cells, transfected 293T and PDEF were seeded onto gelatin-coated plates, at a density of 1x10^5 cells/well and cultured in a 37°C incubator for 2-3 days until they were fully confluent. Monolayers of cells were washed twice with cold PBS, and fixed with 1 mL/well of pre-chilled 95:5 ethanol: acetic acid (EAA) overnight (O/N) at -20°C. Before staining, the EAA was removed and the monolayer of cells was washed 4 times with PBS, and blocked with 150 µL/well of a 1/50 dilution of normal duck serum (NDS) in PBS for 5 min at RT, in a pre-warmed humid container. After blocking, the NDS was removed and 150 µL/well of primary antibody diluted in 10% NDS in PBS was added. The plate was then placed in a pre-warmed humid container for 1 hr at 37°C and then O/N at 4°C. On the following day, the primary antibodies were removed with gentle suction and the plates were washed 4 times using PBS. The monolayer of cells were then incubated with 150 µL/well of the appropriate fluorophore-conjugated secondary antibodies diluted in 10% NDS in PBS for 30 min at 37°C. Excess secondary antibodies were again removed and the cells were washed as described above. Lastly, the cell monolayers were mounted in ProLong® Gold anti-fade reagent (Cat. # P36930, Invitrogen) to suppress photobleaching and maintain fluorescent signals. In addition, ProLong® Gold anti-fade reagent also counterstained the nucleus of the cells with the blue
fluorescent stain DAPI (4',6-diamidino-2-phenylindole). All primary and secondary antibodies used for IMF assays are listed in Table 2-2.

The IMF stained monolayers were visualized and imaged using a Nikon ECLIPSE Ti-E inverted microscope equipped with a high-sensitivity cooled CCD digital camera DS-Qi1 (Nikon). The images were merged and stored for further analysis using the NIS-Elements imaging software developed by Nikon.

2.2.7 Detection of DHBV antigens using Western Blot

Transfected cells grown in 6-well-plates as previously described in Section 2.2.3 were washed with PBS and were lysed with lysis buffer on ice. The cells were then collected using a cell lifter (Cat#3008, Corning) and transferred to an eppendorf tube followed by incubation on ice for 30 min. After centrifugation at 13000 g for 10 min, 75 μL of the clarified supernatant (the cell lysate) was added to 15 μL of 6 x LUG loading buffer (Table 2-3) and denatured at 90°C for 10 min. The samples were kept on ice for 10 min and either directly applied to Western Blot or transferred to -20°C for future use.

Western Blot was performed using a Bio-Rad apparatus (Cat. # 165-80000, Bio-Rad). The Sodium Dodecyl Sulphate (SDS) – Polyacrylamide Gel Electrophoresis (PAGE) gel consisted of a 5% stacking gel top layer and 12% resolving gel bottom layer which was prepared as described in Table 2-3 and allowed to set for 15 min. Then 20 μL of denatured cell lysate (containing ~40-60 ug of protein/well) was loaded onto each lane of the SDS-PAGE gel which was electrophoresed in 1 x running buffer (Table 2-3) for 1 hr at 200V. Following electrophoresis the gel was electro-blotted onto a nitrocellulose (NC) membrane (Cat. # 66485, Pall Corporation) in 1 x transfer buffer for 60-90 min at 200 mA. After electro-blotting, the NC membrane was stained with Ponceau S solution (Cat. # P-7170, Sigma-
Table 2-2: Details of antibodies used in IMF assays to detect specific antigens

<table>
<thead>
<tr>
<th>Target antigen</th>
<th>Primary antibodies</th>
<th>Secondary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Description</td>
</tr>
<tr>
<td>DHBcAg</td>
<td>R39408&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rabbit anti-DHBcAg polyclonal antibodies</td>
</tr>
<tr>
<td></td>
<td>CQT-1, CQT-2</td>
<td>Rabbit anti-DHBcAg polyclonal antibodies</td>
</tr>
<tr>
<td>DHBsAg</td>
<td>1H.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mouse anti-DHBpre-S/S monoclonal antibodies</td>
</tr>
</tbody>
</table>

Note: All primary and secondary antibodies were diluted in PBS

References

<sup>a</sup>(Jilbert et al., 1992)
<sup>b</sup>(Pugh et al., 1995)
Table 2-3: A summary of reagents and solutions used in various assays

<table>
<thead>
<tr>
<th>Western blot</th>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA</td>
<td></td>
</tr>
<tr>
<td>12% SDS-PAGE gel</td>
<td><strong>5% stacking gel (2.5 mL)</strong></td>
<td>425 μL 30% acrylamide/bis solution (Cat. # 161-0158, Bio-Rad), 300 μL 1M Tris (pH 6.8), 25 μL 10% APS (w/v), 25 μL 10% SDS, 5 μL TEMED (Cat. # 161-0800, Bio-Rad), 1.735 mL DW</td>
</tr>
<tr>
<td>12% resolving gel (10 mL)</td>
<td>4 mL 30% acrylamide/bis solution, 2.5 mL 1.5M Tris (pH 8.8), 200 μL 10% (w/v) APS, 100 μL 10% SDS, 20 μL TEMED, 3.34 mL DW</td>
<td></td>
</tr>
<tr>
<td>6 x LUG loading buffer</td>
<td>0.35 mM Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.012% bromophenol blue</td>
<td></td>
</tr>
<tr>
<td>1 x running buffer</td>
<td>3.06 g/L Tris, 14.4 g/L glycine, 1 g/L SDS</td>
<td></td>
</tr>
<tr>
<td>1 x transfer buffer</td>
<td>3.06 g/L Tris, 14.4 g/L glycine, 5% (v/v) methanol</td>
<td></td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>325 μL mercaptoethanol, 8 mL 10% SDS, 1.25 mL 1M Tris (pH 6.8), 30.45 mL DW</td>
<td></td>
</tr>
<tr>
<td>Ponceau S solution</td>
<td>0.1% (w/v) Ponceau S, 5% (v/v) acetic acid, DW</td>
<td></td>
</tr>
<tr>
<td>Coomassie blue staining</td>
<td>Name</td>
<td>Composition</td>
</tr>
<tr>
<td>Coomassie Blue</td>
<td>1% (v/v) Coomassie blue, 45% (v/v) methanol, 5% (v/v) acetic acid, DW</td>
<td></td>
</tr>
<tr>
<td>Destaining buffer</td>
<td>10% (v/v) methanol, 5% (v/v) acetic acid, DW</td>
<td></td>
</tr>
<tr>
<td>Agarose gel electrophoresis</td>
<td>Name</td>
<td>Composition</td>
</tr>
<tr>
<td>10 x TAE DNA loading buffer</td>
<td>60% sucrose, 1% sarkosyl, 1 x TAE buffer, 0.1% bromophenol blue, 0.1% xylene cyanol</td>
<td></td>
</tr>
<tr>
<td>10 x TAE running buffer</td>
<td>400 mM Tris-acetate, 10 mM EDTA</td>
<td></td>
</tr>
<tr>
<td>Other solutions</td>
<td>Name</td>
<td>Composition</td>
</tr>
<tr>
<td>PBS</td>
<td>140 mM NaCl, 3 mM KCl, 1 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$</td>
<td></td>
</tr>
<tr>
<td>Luria Broth (LB)</td>
<td>10 g/L bacto tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl</td>
<td></td>
</tr>
<tr>
<td>20 mM Phosphate buffer (PB) pH 6.8</td>
<td>3.978 gm NaH$_2$PO$_4$ (Cat. # 10245CC, BDH), 3.478 gm Na$_2$HPO$_4$ (Cat. # 102484A, BDH), 5 L DW</td>
<td></td>
</tr>
</tbody>
</table>
Aldrich) to detect the presence of protein. Subsequently, the NC membrane was washed with distilled water (DW), blocked with 5% (w/v) skim milk (Diploma instant, Fonterra brands Pty. Ltd.), in 0.1% Tween 20 (Cat. # P-1379, Sigma-Aldrich) in PBS (0.1% PBS-T) for 30 min at RT, followed by incubation with appropriate primary antibody in 0.1% PBS-T at 4°C, O/N on a shaker. The next day, excess primary antibodies were rinsed off by 3 washes of 0.1% PBS-T followed by the incubation with HRP-conjugated secondary antibodies in 0.1% PBS-T for 1 hr at RT on a shaker. Excess secondary antibodies were rinsed off by 3x15 min washes with 0.1% PBS-T on a shaker. The NC membrane was then treated using Amersham ECL PLus™ Western Blotting Detection Reagents (Cat. # RPN2132, GE Healthcare) for 5 min and dried with paper towel. A sheet of Kodak BioMax MR-1 film (Cat. # 8701302, Kodak) was exposed to the NC membrane for varying times from 5 sec to 15 min before developed in an automated AGFA CP 100 X-ray film processor.

To investigate multiple proteins on the same NC membrane, stripping buffer was used to remove primary and secondary antibodies from the membrane. The membrane was washed in stripping buffer (Table 2-3) at 50°C for 30 min and was further washed in the same buffer at RT for 10 min. The stripping buffer was removed and the membrane was washed 3x15 min in 0.1% PBS-T at RT. The membrane was then blocked with 5% skim milk in PBS-T and re-probed with appropriate primary and secondary antibodies. Details of specific antibodies, reagents and buffers used for all Western Blots are listed in Tables 2-3 and 2-4. Coomassie blue staining (Cat. # 27815, Sigma-Aldrich) was also used to visualise protein polypeptides separated by SDS–PAGE by staining the gel at RT for 30 min followed by washing in a de-staining solution (Table 2-3) at RT for 4 hr on a shaker. Coomassie blue stained gels were visualized on a light box and photographed using a digital camera. Details of specific reagents and buffers used for Coomassie blue staining are listed in Table 2-3.
Table 2-4: Details of antibodies used in Western Blot to detect specific antigens

<table>
<thead>
<tr>
<th>Specific antigen</th>
<th>Primary antibodies</th>
<th>Secondary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Description</td>
</tr>
<tr>
<td>DHBcAg</td>
<td>R39408&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rabbit anti-DHBcAg polyclonal antibodies</td>
</tr>
<tr>
<td>DHBcAg</td>
<td>CQT-1, CQT-2</td>
<td>Rabbit anti-DHBcAg polyclonal antibodies</td>
</tr>
<tr>
<td>DHBcAg</td>
<td>5B-11F</td>
<td>Mouse anti-DHBcAg monoclonal antibodies</td>
</tr>
<tr>
<td>DHBpre-S/S</td>
<td>1H.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mouse anti-DHBpre-S/S monoclonal antibodies</td>
</tr>
<tr>
<td>Beta- lactamase</td>
<td>α-bla</td>
<td>Rabbit anti-beta lactamase polyclonal antibodies (Cat. # AB3738, Millipore)</td>
</tr>
<tr>
<td>Human beta-actin</td>
<td>I-19</td>
<td>Rabbit anti-human beta actin polyclonal antibodies (Cat. # 31460, Santa Cruz Biotechnology)</td>
</tr>
<tr>
<td>Anti-Flag antibodies</td>
<td>M2</td>
<td>Monoclonal anti-FLAG&lt;sup&gt;®&lt;/sup&gt; M2 antibodies (Cat. # F3165, Sigma)</td>
</tr>
</tbody>
</table>

Note: All primary and secondary antibodies were diluted in PBS-T

Western Blot was performed as described in Section 2.2.7.

References
<sup>a</sup>(Jilbert et al., 1992)
<sup>b</sup>(Pugh et al., 1995)
2.3 Production of polyclonal and mABs to recombinant DHBV core antigen (rDHBcAg)

2.3.1 Preparation of rDHBcAg particles

2.3.1.1 Growth of transformed E. coli strains and partial purification of rDHBcAg particles

Cloning of the DHBcAg gene (nucleotides 2647-677, of the USA strain of DHBV, DHBV16; Mandart et al., 1984) into the plasmid pKK233-2 (Clonetech Laboratories, California, USA) to create pKKcore, was performed by J. Summers and R. Lenhoff (Jilbert et al., 1992). Within the pKKcore vector DHBcAg expression occurs under the control of the isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible trc promoter leading to production of rDHBcAg protein molecules that spontaneously assemble into DHBV nucleocapsid-like structures or rDHBcAg particles. The pKKcore vector had been previously transformed into E. coli strain JM101 and was available in the laboratory as a glycerol stock stored at -80°C. To produce rDHBcAg particles, the glycerol stock of E. coli strain JM101 transformed with pKKcore was grown on an agar plate containing 60 mg/mL of Ampicillin at 37°C O/N. The next day, a single colony was picked from the plate and inoculated into 50 mL of Luria Broth (LB) containing Ampicillin followed by incubation at 37°C O/N. When the culture reached an OD of 0.8 at 600 nm, IPTG (Cat. # 11411446001, Roche) was added to a final concentration of 1 mM and the culture was incubated at 37°C O/N to induce production of rDHBcAg. After culture the bacteria were harvested by centrifugation in a JA-10 rotor at 4°C, 6400 g for 20 min and the supernatant was discarded.

The bacterial pellets were then resuspended in 20 mM phosphate buffer (PB) pH 6.8 and the cell suspension was passed twice through a French Press (Aminco, USA), to release the rDHBcAg particles into the supernatant. The collected cell lysate was centrifuged in a JA-20
rotor at 4°C, 7700 g for 10 min to remove particulate debris and the supernatant was collected. rDHBcAg particles and other *E. coli* proteins were then precipitated from the supernatant by adding ammonium sulphate [(NH₄)₂SO₄], to a final concentration of 33.5% and incubating in an ice bath for 1 hr. The precipitated rDHBcAg particles were then collected by centrifugation, at 17,000 g for 15 min at 4°C before being re-dissolved in 20 mM PB pH 6.8 at 4°C and dialysed against the same buffer O/N to remove (NH₄)₂SO₄. After storage at 4°C O/N, a cloudy precipitate was visible. This precipitate, which was later found by SDS-PAGE gel analysis to be contaminating *E. coli* proteins, was removed by centrifugation in a JA-20 rotor, at 4°C, 20,000 g for 15 min. The supernatant was collected in a new tube and the precipitation and dialysis process was repeated 5 times. After the fifth dialysis process, the supernatant, referred to as a crude preparation of rDHBcAg particles, was stored at –20°C before use.

### 2.3.1.2 Separation and isolation of rDHBcAg particles by caesium chloride isopycnic density gradient centrifugation

The preparation of rDHBcAg particles was then purified by caesium chloride (CsCl) isopycnic density gradient centrifugation. The CsCl gradients consisted of 1 mL of 1.7 g/cm³ CsCl (Cat. # C3032, Sigma-Aldrich) overlaid with 3 mL of 1.5 g/cm³, 1.3 g/cm³ and 1.2 g/cm³ of CsCl all dissolved in Tris-EDTA solution. Each gradient was formed in a Beckman SW41 centrifuge tube (Cat. # 344059). The crude preparation of rDHBcAg particles was then applied to the top of the gradient followed by TE until the liquid level reached 1-2 mm from the top of the tube. The tubes were then balanced and centrifuged for 20 hr, at RT, at 21,000 g in a Beckman SW41 swing out rotor in a Beckman Ultracentrifuge (OptimaTM L-100 XP Ultracentrifuge). After centrifugation, 1-2 white bands, which were later identified as rDHBcAg particles, formed in the CsCl gradient, 0.5 mL fractions were collected from the bottom of the tube using a fraction collector and each fraction was subjected to ELISA testing as described below.
2.3.1.3 Detection of rDHBcAg particles by ELISA

An ELISA assay was performed to determine which fractions from the CsCl gradient contained the highest amount of rDHBcAg particles. Costar® 3590 flat bottom 96-well microtitre plates (Corning Incorporated, USA) were divided into two halves and each half of the plate was coated with 100 µL per well of a 1/100 dilution of each fraction in PBS, then 4x-fold dilutions across the plate to a final dilution of 1/62,500. After O/N incubation at 4°C, plates were washed 3 times with PBS-T using an automated plate washer (ELx405 Select, Biotek®, USA) and blocked with 200 µL of 5% skim milk in PBS-T per well at 37°C for 1 hr to block any non-specific binding sites. After washing 3 times with PBS-T, the first half of each plate was incubated with 100 µL per well of 1/5,000 dilution of duck serum containing duck anti-DHBcAg antibodies (Derived from duck # 262 that had been previously infected with DHBV). The second half of each plate was incubated with 100 µL per well of a 1/5,000 dilution of NDS, both diluted in 5% skim milk in PBS-T. Plates were incubated at 37°C for 45 min followed by 3 washes with PBS-T. 100 µL per well of a 1/15,000 dilution of polyclonal rabbit anti-duck IgY antibodies (Bertram, 1997) in 5% skim milk in PBS-T was then added to each well and incubated at 37°C for 45 min. After 3 washes with PBS-T, 100 µL per well of 1/4,000 secondary horseradish peroxidase (HRP) conjugated, Goat anti-rabbit polyclonal antibodies (Kierkegaard Perry Laboratories Inc. Maryland, U.S.A) diluted in 5% skim milk in PBS-T was added. The plates were incubated at 37°C for 45 min followed by 3 washes with PBS without Tween-20. The O-phenylenediamine dihydrochloride (OPD) substrate was prepared by adding 1 tablet of Urea buffer and 1 tablet of OPD (Sigma Cat. # P-9187) to 20 mL of DW. 100 µL per well of the OPD substrate was then added to the plate which was then incubated in the dark for 15 min. The reaction was stopped by adding 50 µL per well of stop solution (2.5 M H₂SO₄). The optical density was then measured at 490 nm using an automated plate reader (Spectra Max M2, Molecular Devices, Millennium Science, USA).
2.3.1.4 Precipitation of rDHBcAg using Trichloroacetic acid (TCA)

Since the presence of CsCl affects protein migration on SDS-PAGE gels, rDHBcAg particles present in each fraction were precipitated using TCA (Cat. # 102865J, BDH) to remove CsCl. 150 μL of each fraction containing rDHBcAg, 175 μL of chilled PBS, and 65 μL of chilled 100% TCA was mixed together and held on ice for 10 min. The mixture was then centrifuged at 11,000 g for 10 min at 4°C. After the supernatant was removed, 300 μL of chilled 1% TCA was added and the TCA-precipitated pellet was again collected after centrifugation at 11,000 g for 10 min at 4°C. TCA precipitated pellets were then resuspended in 1 x LUG buffer and heated at 90°C for 10 min to dissociate the rDHBcAg particles into monomers. The samples were kept on ice for 10 min and transferred to -20°C before being applied to Western Blot or Coomassie blue staining as previously described in Sections 2.2.7 and 2.2.8.

2.3.1.5 To concentrate rDHBcAg particles and to determine the concentration of rDHBcAg particles.

To concentrate the rDHBcAg particles present in each CsCl fraction, Amicon Ultra-15 Centrifugal Filters were used (Cat. # UFC901008, Millipore). Before using, the Amicon filter was washed 2 times with DW and equilibrated with 20 mM PB pH 6.8 (the same buffer used in Section 2.3.1.1) followed by centrifugation at 3600 g for 10 min. After removing the PB buffer, individual fractions containing rDHBcAg particles were pooled together and ~15 mL was applied to each Amicon Ultra-15 Centrifugal Filter which was then centrifuged at 3600 g for 35 min at 4°C. Following centrifugation, 15 mL of PBS was added and the filter was centrifuged at 3600 g for 30 min. This process was then repeated 3 times, to exchange the buffer from 20 mM PB pH 6.8 to PBS. Following the last wash 500 μL of PBS was added to the filter and mixed carefully to resuspend the rDHBcAg particles before 500 μL of concentrated rDHBcAg particles were collected and transferred to a clean eppendorf tube and
stored at -20°C before being used to immunise rabbits and mice as described in Sections 2.3.2 and 2.3.3 below. To determine the concentration of rDHBcAg particles, a Quick Start™ Bradford protein assay (Cat # 500-0201, BIO-RAD) was used as following the manufacturer’s instructions. The final yield of purified rDHBcAg was ~6 mg/L.

2.3.2 Production of polyclonal rabbit antibodies to rDHBcAg

All rabbit handling, injections and blood sampling was performed by staff in the SA Pathology Veterinary Science Division, Gilles Plains, South Australia. Two rabbits were injected by the subcutaneous (s.c.) route in 4-6 sites with a total of 500 μg of purified rDHBcAg particles emulsified in Freund’s complete adjuvant (FCA). Three weeks later, a second dose of rDHBcAg (500 μg) was given via the s.c. route to the rabbits in Freund’s incomplete adjuvant (FIA) and this was repeated (third dose) after a 3 week interval. 10 days after the third dose, an intravenous (i.v.) blood sample was collected from each rabbit to test by ELISA for the presence of serum anti-rDHBc antibodies as described below. Rabbits that showed a high antibody response were given a fourth dose of rDHBcAg in FIA and then 10-14 days later the rabbits were sacrificed using ketamine anesthesia (Parnell Laboratories Australia Pty. Ltd.) and their blood was collected by cardiac puncture (~80 mL) and used to prepare serum for testing in ELISA.

2.3.2.1 Testing antibody responses by ELISA following immunisation with rDHBcAg

Costar® 3590 flat bottom 96-well microtitre plates were coated with 100 μL per well of 10 μg/mL of rDHBcAg (purified as described in Section 2.3.1.1) in 0.1 M NaHCO₃ buffer pH 9.6 and incubated at 37°C for 1 hr followed by 4°C O/N. Plates were washed 3 times with PBS-T using an automated plate washer and blocked with 200 μL of 5% skim milk in PBS-T per well at 37°C for 1 hr. After 3 washes with PBS-T, plates were coated with 100 μL per well of a 1/125 dilution in 5% skim milk in PBS-T of each serum sample collected from the
immunised rabbits or mice. Each sample was then diluted five-fold across the plate. Plates were incubated at 37°C for 1 hr followed by 3 washes with PBS-T. For rabbit sera, 100 μL per well of a 1/4000 dilution of secondary HRP-conjugated goat anti-rabbit polyclonal antibodies (Kierkegaard Perry Laboratories Inc. Maryland, U.S.A) diluted in 5% skim milk in PBS-T was added. For mice sera, 100 μL per well of 1/5000 secondary HRP-conjugated sheep anti-mouse polyclonal antibodies (Cat. # NA9310V, GE Healthcare Limited, UK) diluted in 5% skim milk in PBS-T was added. The plates were sealed and incubated at 37°C for 1 hr. Plates were then washed 3 times with PBS, and OPD and stop solution were then added followed by measuring OD at 490 nm as previously described in Section 2.3.1.3.

2.3.2.2 Absorption of polyclonal antisera with normal duck liver (NDL) and NDS
To remove antibodies that were cross reactive, polyclonal antisera were absorbed with NDL and NDS. Firstly, 18.5 g of NDL was homogenised with a tissue homogeniser in 20 mL of PBS followed by centrifugation in Beckman 55-2 Ti rotor, at 149,000 g for 2 hr at 4°C. After the supernatant was removed, the NDL pellet was re-suspended in 3 mL of NDS and 1.5 mL of polyclonal antisera. The suspension was then incubated at 37°C for 1 hr and was kept at 4°C O/N. The next day, the suspension was centrifuged for 2 hr, at 4°C, at 210,000 g in a Beckman SW41 swing out rotor in a Beckman Ultracentrifuge and ~4.5 mL of absorbed polyclonal antisera was carefully collected taking care to avoid both lipid and red blood cell layers. The absorbed polyclonal antisera were then filtered with a 0.2 μm filter and stored at -20°C.

2.3.3 Production of mABs to rDHBcAg
All mouse handling, injections and blood sampling was performed by staff in the SA Pathology Veterinary Science Division, Gilles Plains, South Australia. 6-week-old BALB/c mice were injected via the intra-peritoneal (i.p.) route with 50 μg of purified rDHBcAg
particles emulsified in FCA. Three weeks later, the mice were injected i.p. with 50 µg of antigen in FIA followed by another two boosting doses of 50 µg without adjuvant at 3 week intervals. 10-14 days after the third dose, a serum sample (~50-100 µL) was collected by retro-orbital bleeding from each mouse to test the antibody titre. Mice that showed higher titres of anti-DHBc antibodies in comparison to pre-bleed serum were given a fourth dose of antigen (25 µg) without adjuvant via the i.p. and i.v. routes and then 3 days later the mice were sacrificed and their spleens were removed and homogenized into single cell suspension as described in Section 2.3.3.1.

2.3.3.1 Hybridoma production.
The complete composition of all reagents and culture media used for the production of mAB are listed in Table 2-5.

**Myeloma cells:** A week before the fusion, SP2/0 myeloma cells were thawed and seeded at 2-3x10⁵ cells/mL in supplemented DMEM and grown to ~8x10⁵ cells/mL in 175 cm² flasks. When needed for fusion, the myeloma cell concentration was adjusted to 1x10⁷ cells/mL in serum-free DMEM.

**Feeder cell preparation:** The unimmunised mice were sacrificed via cervical dislocation and were placed in a beaker containing 70% ethanol. Spleens were then removed using aseptic technique and transferred to a petri dish containing serum-free GI 1640 (Cat. # A10491-01 Gibco). The spleens were then cut finely using a sterile scalpel blade and homogenised using a tissue homogeniser. Debris was removed by filtration through pre-wet cotton wool by centrifugation at 900 g for 10 min at 26°C. The supernatant was removed and the cell pellet was resuspended in fusion medium (see Table 2-5) leading to a single cell suspension. The cell concentration was adjusted to 4x10⁶ cells/mL. The feeder cells were incubated at 37°C water bath until needed for the fusion process.
Table 2-5: A summary of reagents and culture media used in the production of monoclonal antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion medium</td>
<td>40% DMEM, 40% Gl1640, 20% FCS, 1.2 mg/L penicillin, 16 mg/L gentamycin</td>
</tr>
<tr>
<td>HA medium</td>
<td>150 mL Fusion medium, 1.5 mL 100 x Hypoxanthine (Cat. # H9377-25G, Sigma-Aldrich), 1.5 mL 100 x Azaserine (Cat. # A1164-5MG, Sigma-Aldrich)</td>
</tr>
<tr>
<td>Cloning medium</td>
<td>20% FCS, 20% MAbSA cloning medium, 30% DMEM, 30% Ham’s F12 (Cat. # 11765-054, Gibco)</td>
</tr>
<tr>
<td>100 x Hypoxanthine</td>
<td>1.36 g/L Hypoxanthine, DW</td>
</tr>
<tr>
<td>100 x Azaserine</td>
<td>0.1 g/L Azaserine, DW</td>
</tr>
<tr>
<td>Freezing medium</td>
<td>10% DMSO, 20% FCS, 70% DMEM</td>
</tr>
</tbody>
</table>

The production of monoclonal antibodies was performed as described in Section 2.3.3.
**Preparation of spleen cells from immunised mice:** The preparation of spleen cells from immunised mice was similar to the preparation of the feeder cells. Immunised mice were sacrificed, their spleen was removed, and homogenised, filtered and centrifuged. After the supernatant was discarded, the cell pellet was washed twice with serum-free GI 1640, collected by centrifugation at 900 g for 10 min and the cell concentration was adjusted to 2x10^7 cells/mL.

**Cell Fusion:** The spleen cell suspension was mixed with SP2/0 cells in a 5:1 ratio then centrifuged at 900 g for 10 min. The supernatant was removed, except for 500 µL in which the cell pellet was resuspended and spun again. After the supernatant was removed, 1 mL of sterile poly-ethylene-glycol (PEG) 1500 (Cat. # 81210, Sigma-Aldrich) was added drop wise over 1 min and the cell pellet was continuously stirred gently to mix the cells. The cells were incubated for 1 min at 37°C and then cell mixture was slowly diluted with 2 mL serum-free GI 1640 over 2-3 min with constant stirring. The cells were centrifuged at 700 g for 5 min and the supernatant was removed. The pellet was resuspended with Fusion medium and the cell concentration was adjusted to 4x10^6 cells/mL. Equal volume of feeder cell suspension was then added to the fusion cell suspension.

**Plating:** The fusion-feeder cell suspension was then plated at 1 mL/well into 24-well-plates or at 100 µL/well into 96-well round bottom plates and kept at 37°C in an incubator supplied with 5% CO₂. The next day, HA medium (Table 2-5) was added to 24-well-plates at 1 mL/well or 96-well-plates at 100 µL/well. At days 7 and 10 of culture, half of the medium from each well was aspirated and replaced with fresh HA medium. Fusion cells appearing as clumps were observed from day 10 onwards. When the medium turned to yellow in color, hybridoma supernatants were screened for anti-DHBc antibodies using the ELISA assay described in Section 2.3.3.2. Hybridomas producing the anti-DHBc mABs were expanded in
cloning medium and were cloned by limiting dilution 2 times. These positive clones were cryo-preserved with freezing medium ((Table 2-5) at 5x10^6 cells/mL. The selected positive clones were sent to Monoclonal Antibodies SA (MAbSA) for large-scale mAB production and purification by protein G affinity chromatography.

**2.3.3.2 Hybridoma screening for anti-DHBc antibodies**

The neat hybridoma supernatant was collected and screened for anti-DHBc antibodies by ELISA as described in Section 2.3.2.1. A 1/10 dilution of selected hybridoma supernatants were screened for anti-DHBc antibodies against lysates of the Dstet5 cell line, against proteins extracted from DHBV-infected duck liver and against purified rDHBcAg in Western Blot as described in Section 2.2.5.

**2.4 Procedure involving Duck CD40L (DuCD40L)**

All techniques described below in Sections 2.4.1 – 2.4.2 were performed by Dr Feng Feng in the Hepatitis Virus Research Laboratory and are presented here for completeness.

**2.4.1 Sub cloning of the DuCD40L gene into the pcDNA3 expression vector**

**2.4.1.1 Plasmid vector and primer design**

The pcDNA3 expression vector (Invitrogen, Carlsbad, CA) comprising an Ampicillin resistance gene and a multiple cloning site downstream of a CMV promoter was used as a cloning vector to express the DuCD40L gene. Primers for DuCD40L gene amplification were designed using the internet based program, Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The DuCD40L nucleotide sequence used for PCR primer design was based on the reported DuCD40L sequence (GenBank Accession DQ267671), from the GenBank Database at the National Centre for

2.4.1.2 RNA extraction

Total RNA was isolated from duck PMBC and splenocyte cultures, either untreated or stimulated with PHA or ConA, using TRIZOL® reagent (Cat. # 15596-02, Invitrogen). In brief, to extract cellular RNA, the culture supernatant was removed before the addition of TRIZOL® reagent (~300-500 µL/well in 24-well-plates) and incubation at RT, for 5 min. An equal volume of AR grade chloroform (Gibco Life Technologies, USA) was added to the mixture which was then shaken vigorously for 15 sec followed by incubation at RT for 3 min. The lysate/chloroform mixture was centrifuged for 15 min at 12,000 g at RT and the aqueous layer was transferred to a clean eppendorf tube. In the eppendorf tube, 100 µL of isopropanol was added and the mixture was incubated at RT for 10 min. After centrifugation for 10 min at 12,000 g at RT, the supernatant was removed and 200 µL of 75% ethanol was added and centrifuged for 5 min at 7000 g at RT. The supernatant was discarded and the pellet was air-dried and redissolved in RNase-free DW. DNA in the RNA isolate was removed using a DNase I RNase-Free Kit (Cat. # 2222, Ambion) and the purified RNA was stored at -20°C. The purity and concentration of each RNA sample was determined by measuring absorbance at 260 nm and 280 nm using a Nanodrop1000 spectrophotometer (Thermo scientific).

2.4.1.3 The cDNA synthesis and cloning by PCR

First-strand DuCD40L cDNA was synthesized from 2 µg of extracted RNA using Omniscript RT Kit and oligo-dT primer (Cat. # 205110, Qiagen). PCR amplification was performed using the Promega Pfu® PCR system (Cat. # M776A, Promega). Each 50 µL reaction mix contained 5 µL of 10X Pfu reaction buffer with MgSO₄, 5 µL of 2 mM dNTP mix, 2 µL of 10 µM forward and reverse DuCD40L primers (Table 2-6), 1.25 units of Pfu DNA polymerase (Cat. # M774A, Promega), 5 µL of cDNA template and sterile DW. The reactions were performed
Table 2-6: Details of PCR primers and their sequences for specific targets

<table>
<thead>
<tr>
<th>Target</th>
<th>Original GenBank Accession #</th>
<th>Primer pair</th>
<th>Primer position</th>
<th>PCR product size (bp)</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DuCD40L</td>
<td>DQ267671</td>
<td>CD40L(F)</td>
<td>61-74</td>
<td>820</td>
<td><strong>CAGGATCCGGCCACCATGAATGAAGITCTA</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD40L(R)</td>
<td>879-858</td>
<td></td>
<td><strong>CAGTGTCTTGCTGCCAACAG</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iNOS(F)</td>
<td>39-58</td>
<td>229</td>
<td><strong>TGATCTTTTGCTGCCAACAG</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iNOS(R)</td>
<td>268-248</td>
<td></td>
<td><strong>GCTGTTCACACACGGGAA</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>iNOS</td>
<td>GGU34045</td>
<td>iNOS(F)</td>
<td>390-410</td>
<td>276</td>
<td><strong>CAGATCCTCCCTGCTAGGA</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>iNOS(R)</td>
<td>666-646</td>
<td></td>
<td><strong>ATTGCCCTATGCTGACAT</strong></td>
</tr>
<tr>
<td>AusDHBV</td>
<td>AJ006350</td>
<td>390</td>
<td>390-410</td>
<td>276</td>
<td><strong>CTAAGCATCACCTGGGAAA</strong></td>
</tr>
<tr>
<td>total DNA</td>
<td></td>
<td>666c</td>
<td>666-646</td>
<td></td>
<td><strong>GCTGGGACAAATTTTCGCA</strong></td>
</tr>
<tr>
<td>AusDHBV</td>
<td>AJ006350</td>
<td>423&lt;sup&gt;c&lt;/sup&gt;</td>
<td>423-442</td>
<td>154</td>
<td><strong>CCTGATTGGAGGCTTCTTAC</strong></td>
</tr>
<tr>
<td>total DNA</td>
<td></td>
<td>576&lt;sup&gt;c&lt;/sup&gt;</td>
<td>576-556</td>
<td></td>
<td><strong>AAAGGTACAGTCAGGCTG</strong></td>
</tr>
<tr>
<td>AusDHBV</td>
<td>AJ006350</td>
<td>CC2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2462-2481</td>
<td>156</td>
<td><strong>CCTGATTGGAGGCTTCTTAC</strong></td>
</tr>
<tr>
<td>cccDNA</td>
<td></td>
<td>MG1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2618-2599</td>
<td></td>
<td><strong>AAAGGTACAGTCAGGCTG</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup>: The DuCD40L nucleotide sequence used for PCR primer design was based on the reported duck CD40L sequence (GenBank Accession DQ267671). The sequence of the upstream primer of DuCD40L which contains a *Bam*HI RE digestion site (underlined) and a Kozak sequence GCCACC (shown in bold).

<sup>b</sup>: The DuCD40L nucleotide sequence used for PCR primer design was based on the reported duck CD40L sequence (GenBank Accession DQ267671). The sequence of the downstream primer of DuCD40L which contains an *Xho*I RE digestion site (underlined) and a Flag epitope sequence (shown in bold).

<sup>c</sup>: The primer set (423-576c) was designed to bind to the polymerase ORF of the DHBV genome, downstream of the core ORF and upstream of the pre-S/S ORF (Reaiche et al., 2010). Therefore, the primers will not amplify DHBV C, S and pre-S/S sequences present in the DHBV-DNA vaccines and rFPV-DHBV vaccines.

<sup>d</sup>: The cccDNA primer set (CC2-MG1) was designed to bind specifically to the cohesive overlap region of the DHBV rcDNA. When the double-stranded cccDNA is denatured, two complete circular strands can be amplified by the CC2-MG1 primer set (Reaiche et al., 2010).
in a Thermo cycler (Perkin Elmer, GeneAmp PCR system 2400). The PCR reaction was first denatured at 95°C for 3 min followed by 30 cycles of 40 sec DNA denaturation at 95°C, 40-sec primer annealing at 59°C, and 3 min primer extension at 72°C, followed by final extension at 72°C for 5 min. The PCR product was analyzed by mixing each DNA sample with 10 X TAE loading buffer (Table 2-6), and electrophoresis on 1% agarose gels in 1 X TAE buffer (40 mM Tris Acetate, 1mM EDTA, pH 7.5) at 90 volts for 30-45 min. Gels were then stained with Gel Red solution (Biotium, USA) at RT for 20 min and visualized using a Gel-Doc XR imaginer (Cat. # 170-8190, Bio-Rad). Gel containing the DNA fragment of interest was excised and the DNA was purified using QIAquick® Gel Extraction Kit (Cat. # 28704, QIAGEN).

2.4.1.4 Restriction Endonuclease (R.E.) digestion
The PCR sample was digested with 2-4 units of RE (New England Biolabs) in a total volume of 20-60 μL reaction that contained 1X manufacturer’s recommended NEB reaction buffer, 1X bovine serum albumin (BSA, 0.1 mg/mL), and sterile DW. All reactions were performed at 37°C for 1 hr or up to O/N before analysis by agarose gel electrophoresis to confirm that the digestion was completed.

2.4.1.5 Ligation
To ligate DNA and subsequently subclone the amplified DuCD40L PCR product into BamHI and XhoI sites of pcDNA3 downstream of the CMV promoter, vector and insert DNA (1:2 molar ratio) were ligated at 4°C O/N in a mixture containing 1 μL of T4 ligase (Cat. # M0202S, NEB), 2 μL of 10X T4 ligase buffer (NEB) and sterile DW, giving a total volume of 20 μL per reaction. The samples were then examined by agarose gel electrophoresis to confirm that the ligation was complete.

2.4.1.6 Transformation
10ng of plasmid DNA or 2 μL of ligation reaction was mixed with 100 μL competent DH5α E. coli cells and were kept on ice for 30 min. The competent cells were heat shocked at 42°C for 90 sec followed by 5 min incubation on ice. To allow recovery of the competent cells, 900μL of super optimal culture (SOC) medium was added and incubated at 37°C for 30 min followed by centrifugation at 3000 g for 4 min. The pellet was then resuspended in 100 μL of SOC medium before spreading onto agar plate containing Ampicillin and incubated O/N at 37°C. Colonies grown on LB-Amp plates were aseptically inoculated into 10 mL of LB-Amp medium. After incubation at 37°C for 8 hr, 500 μL of bacteria culture in late exponential phase growth was mixed with 300 μL of 100% sterile glycerol. 200 μL of bacteria glycerol stock was kept in a screw-capped tube and stored at -80°C.

2.4.1.7 Preparation of plasmid DNA
Small scale plasmid purification was performed using QIAprep Spin Miniprep Kit (Cat. # 27104, QIAGEN). For large scale plasmid DNA purification, JETstar 2.0 Plasmid Purification MAXI Kits (Cat. # 220020, Astral Scientific) were used. To prepare large scale plasmid DNA, glycerol cultures stored at -80°C were thawed and small amount was transferred to 10 mL LB-Amp medium using a sterile loop. The bacterial culture was incubated for ~8 hr with agitation at 37°C then transferred to 500 mL of LB with 100 μg/mL Ampicillin followed by incubation O/N with agitation at 37°C. The 500 mL bacterial culture was transferred to 2, 250 mL centrifuge tubes and spun at 5500 g, for 10 min at 4°C to pellet the cells. The supernatant was removed and 12 mL of suspension buffer was added to the pellet and mixed until the suspension was homogeneous. 10 mL of cell lysis buffer was added to the homogenous re-suspended cells and gently mixed until the lysate appeared homogenous. The lysate was incubated at RT for 5 min. Neutralization buffer was added, samples were inverted immediately until the viscous matter had disappeared after which the mixture was transferred to Oakridge tubes and spun at 39,000 g, 18°C for 30 min. Resulting
supernatants were transferred to falcon tube to remove floating debris before loading onto a column that had been equilibrated with 30 mL of equilibrium buffer. When supernatants were run through, columns were immediately washed with 60 mL of washing buffer. After the washing buffer was run through, plasmid DNA was eluted from the column into an Oakridge tube by adding 15 mL of elution buffer. Collected plasmid DNA was precipitated by adding 10.5 mL of isopropanol and mixed well followed by centrifugation at 39,000 g, 4°C for 30 min. Supernatant was removed, the pellet was washed using 1 mL of 70% ethanol and transferred to 1.5 mL eppendorf tube. The tube was spun at 27,000 g at 4°C for 10 min. Supernatant was poured off and the pellets were air dried at 37°C for ~15-20 min. DNA pellets were re-dissolved in 100 µL of sterile DW and vortexed. Plasmid DNA concentration was determined by a Nanodrop1000 spectrophotometer and stored at -20°C. The final yield of purified plasmid DNA was ~500-800 µg per 500 mL culture.

2.4.2 Nitrate assay

The ability of DuCD40L inducing HD11 cells to produce nitric oxide (NO) was determined by assaying nitrate accumulation as described by Huang et al., 2001. Supernatants (100 µL) from pcDuCD40L or pcDNA3 plasmids DNA transfected LMH cells was collected and added to separate wells seeded with 2x10⁵ HD11 cells before plates were incubated at 37°C with 5% CO₂. After 24 hr or 48 hr post-incubation, culture supernatant (100 µL) was collected and mixed with an equal volume of Griess reagent followed by 10-15 min incubation time at RT. The nitrate production was assayed with Griess reagent in 96-well microtitre plate (Corning Incorporated, USA). The Griess reagent was prepared by mixing the equal volumes of 1% sulphanilamide in 2.5% H₃PO₄ and 0.1% naphthylethylene diamine. The optical density of the wells was determined at a wavelength of 540 nm. The levels of nitrate in each sample was calculated from a standard curve generated using serial two-fold dilutions of sodium nitrite.

2.4.3 Statistical analysis
To determine the statistical significance of the difference in data obtained in the NO assay, the
*p*-value was calculated using the one way ANOVA by Prism software. The *p*-value of <0.05
is considered statistically significant.

2.4.4 Quantitative reverse transcription PCR (qRT-PCR) to detect iNOS mRNA

To measure levels of iNOS mRNA in the RNA sample extracted from HD11 cells as
described in Section 2.4.1.2, RT-qPCR was performed in an AB StepOnePlus™ Real Time
PCR machine (Applied Biosystems). Each reaction contained 10μL of SYBR green PCR
master mix, 5μL of template cDNA, 0.6μL of 10 μM forward and reverse primers specific for
iNOS (Table 2-6), and 3.8μL of dH2O. Reactions were performed in duplicates in MicroAmp
Optical 8-tube strips (Applied Biosystems (AB), USA, catalogue no. 4316567 or 4358293)
capped with optical caps (AB, USA). The following thermocycling conditions were applied:
2-min denaturation at 50°C, 10-min polymerase activation at 95°C, and 40 cycles of
amplification at 95°C for 15 sec and at 60°C for 1 min. Samples from non-transfected HD11
cells were used as a negative reference, and qPCR specific for the β-actin (Table 2-6) was
carried out in parallel on all samples as endogenous normalisation controls. Relative quantity
of targets in the samples were determined by the StepOnePlus™ Systems software, by
comparing normalised target quantity in each sample to normalised target quantity in non-
transfected HD11 cells.

2.5 Procedures involving ducks

2.5.1 DHBV stock and intravenous inoculation of ducks

The DHBV stock was derived from a pool of DHBV-positive serum isolated from ducks
congenitally infected with the Australian strain of DHBV (AusDHBV) (Triyatni et al., 2001).
The Pool (Pool 8) has been previously shown to contain ~5x10⁹ DHBV DNA genomes per
mL and 50 μg/mL of DHBsAg (Jilbert et al., 1996). Virus inoculation was administered i.v.
via the jugular vein using a 29-gauge (G) needle with an insulin syringe. Prior to inoculation, the skin and feathers overlaying the jugular vein were disinfected with 70% ethanol.

2.5.2 DHBV vaccine and vaccination protocol

2.5.2.1 DHBV vaccines

Two different types of DHBV vaccines were used in the studies described in this thesis; DNA vaccines and rFPV vaccines. The DHBV DNA vaccines expressing DHBV core (C), pTC-Dcore, were previously provided by Dr. Fritz von Weizacker from the University Hospital, Freiburg, Germany (Von Weizsacker et al., 1995). The DHBV DNA vaccines expressing either the DHBV surface (S) or pre-surface (PS) were cloned into pcDNA1.1Amp as previously described (Triyatni et al., 1998). These plasmids were used as a positive control in Chapter 3.

To increase the efficiency of DHBV DNA vaccine in this study, DHBV C, S and pre-S/S genes were recently cloned into plasmid pcDNA3 (Invitrogen, Carlsbad, CA) individually as described (Feng et al., 2010) and were used in studies in Chapters 3, 4, 5, and 6.

rFPV vaccines

The rFPV strains expressing DHBV antigens, rFPV-DHBV, were produced as previously described (Miller et al., 2008) by cloning the DHBV C and DHBV pre-S/S genes into shuttle vector pAF09 followed by homologous recombination of pAF09 with the FPV vaccine strain, FPV-M3 and selecting rFPV strains that were resistant to mycophenolic acid and expressed DHBV antigens. Recombinant FPV-M3 strains were checked for insertion of the DHBV C and pre-S/S genes by PCR and positive clones were expanded by inoculation of rFPV-DHBV into chicken embryonic skin cells (CESC) followed by harvesting the cell lysates. Cloning of the DHBV genes into plasmid pAF09 was performed by Dr Darren Miller (University of
Adelaide) and derivation of rFPV-DHBV strains was performed by David Boyle (CSIRO, Geelong) as previously described (Miller et al., 2008). The two separate rFPV-DHBV vaccines, rFPV-DHBc (rFPV-124) and rFPV-DHBpre-S/S (rFPV-125) were selected that encode the DHBV C and DHBV pre-S/S genes respectively. Both DHBV DNA vaccine and rFPV-DHBV vaccines were delivered to ducks by i.m. injection.

2.5.2.2 Vaccination protocols
Before i.m. injection, the thigh muscle area was disinfected with 70% ethanol. The DHBV DNA vaccines (Chapter 5: 250 µg each in 4-day-old ducks; Chapter 4 and 6: 500 µg each in 6-week-old and 14-day-old ducks respectively) and DuCD40L expression construct (Chapter 5: 250 µg each in 4-day-old ducks; Chapter 4: 500 µg each in 6-week-old) were delivered i.m. using a 27G needle with an BD Ultra-Fine™ insulin syringe (Cat. # 326769) into the thigh muscle during the first two weeks of vaccination followed by a 23G needle with 1 mL syringe during the remainder of vaccination. Ducklings were also inoculated i.m. in the thigh muscle with 100 µL of each undiluted stock rFPV-DHBV vaccines [5x10^8 plaque forming units (p.f.u.)/mL] using a 23G needle and a 1 mL syringe.

2.5.2.3 Administration of antiviral drugs
The antiviral drug Entecavir (ETV) was kindly supplied by Bristol-Myers Squibb Pharmaceuticals, USA, in powdered form. ETV was dissolved at 1 mg/mL in sterile DW at 42°C for 1 hr with agitation and stored at 4°C for a maximum of 3 days as previously described (Foster et al., 2003, 2005, Miller et al., 2008). Daily oral treatment of drug was administrated to ducks at a dose of 1 mg/kg body weight by using a 2 mL cuff-less oral/nasal tube attached to a 3 mL syringe (1.0 mg/kg/day).

2.5.4 Venipuncture
All blood samples were collected by venipuncture of the jugular vein. Prior to collection the neck was swabbed with 70% ethanol and blood was collected using a 23G needle with a 3 mL syringe then placed into a 1.5 mL centrifuge tube. After the needle was removed pressure was applied to the vein to minimize the bruise and to stop bleeding. To collect serum from the blood samples, the tubes were incubated at 37°C for 1 hr followed by 4°C O/N. Next day, blood samples were spun at 4500 g for 10 min to separate the serum from the clot. The serum fraction was then transferred to a clean eppendorf tube and stored at -20°C.

2.5.5 Liver biopsy

Prior to biopsy, all instruments were autoclaved and the ducks were fasted by withdrawing feed the night before surgery. The ducks were placed on a heated pad on their back during the surgery. The ducks were anesthetized with 5% isofluorane (Forthane, Abbott, USA) in 6% oxygen with a balloon placed directly over the head of the duck. During the biopsy anesthesia was maintained by ventilation the ducks with 3% isofluorane via an endotracheal tube (size 2.0, ContourTM Mallinckrodt Medical, Ireland). The feathers of the lower abdomen were trimmed with electric clippers and the area was disinfected with betadine and 70% ethanol. The ducks were administered with 300 μL of 0.5% of Bupivacaine hydrochloride (Pharmacia and Upjohn) at the incision site. A 2 cm incision was made and a tissue clamp used to extract a section of liver ~400 mg and cut with a scalpel blade. The incision was closed using 4-0 Vicryl sutures (Ethicon, USA). After surgery, anesthetic was removed and the ducks regained consciousness and were able to stand within a few minutes. As post-operative treatment, ducks were given 5 mg/kg of Ketoprofen (Troy Laboratories Pty. Ltd., Australia) into the thigh muscle.

2.5.6 Autopsy
To euthanize ducks prior to autopsy ducks were injected i.v. with 3-5 mL of barbiturate anesthetic (Lethabarb®, Virbac, Australia). Blood was collected through cardiac puncture for ELISA and for DHBV DNA extraction prior to quantitative PCR (qPCR). The abdominal cavity was opened and both lobes of the liver were removed and sliced using sterile a scalpel blade and a clean petri dish so that liver samples (~0.5 cm$^3$) were collected from 4-5 sites throughout the liver. Samples of pancreas, spleen and kidney tissues (~0.5 cm$^3$) were also collected. All tissues were fixed in ethanol-acetic acid (EAA) or 10% formalin as described in the Section 2.5.7 below. The liver tissues were also snap-frozen in liquid nitrogen and then transferred to -80°C in screw-capped eppendorf tubes.

2.5.7 Tissue fixation, embedding and sectioning

Biopsy and autopsy samples of duck liver, kidney, spleen and pancreas were placed in tissue embedding cassettes (Techno-Plas, Australia). For EAA fixation, the samples were immersed in EAA solution (3:1 v/v) for 30 min at RT. The samples were then washed twice with cold 70% ethanol and kept in 70% ethanol at 4°C O/N. For formalin fixation, samples were immersed in 10% formalin at RT O/N. The EAA- and formalin-fixed tissues were wax embedded, and sectioned at 6 μm onto glass slides in the Division of Tissue Pathology or Department of Neuropathology at SA Pathology. Sections from EAA-fixed tissues were subjected to immuno-staining for DHBsAg as described in Section 2.7.2. Sections from formalin-fixed tissues of each duck were stained using H&E for histological analysis or were subjected to immuno-staining for DHBcAg as described in Section 2.7.3.

2.6 Serological Assays

ELISAs developed by Dr. Darren Miller (Miller et al., 2004) were used in this project to assess the levels of serum DHBsAg, anti-DHBs and anti-DHBc antibodies.

2.6.1 Quantitative detection of DHBsAg by ELISA
Costar® 3590 flat bottom 96-well micro-titre plates (Corning Incorporated, USA) were coated in duplicate with 100 µL samples of each test serum sample diluted 1/100 in PBS. Plates were sealed and incubated at 37°C O/N. Plates were washed 3 times with PBS-T using an automated plate washer. 200 µL per well of 5% skim milk in PBS-T was added and incubated at 37°C for 1 hr to prevent non-specific binding. After washing 3 times with PBS-T, 100 µL per well of 1/5000 primary anti-DHBV pre-S/S mABs, 1H.1 (Pugh et al., 1995) diluted in 5% skim milk in PBS-T was added and plates were once again sealed and incubated at 37°C for 1 hr. Plates were washed 3 times with PBS-T followed by adding 100 µL per well of 1/5000 secondary HRP-conjugated sheep anti-mouse polyclonal antibodies (Cat. # NA9310V, GE Healthcare Limited, UK) diluted in 5% skim milk in PBS-T. Plates were again sealed and incubated at 37°C for 1 hr before washing with PBS and developing with OPD as previously described in Section 2.3.1.3.

For each plate, a standard curve was established using eight, 2-fold dilution of Pool 8 serum (1/500 to 1/64000) in 1 x PBS. The NDS wells and blank wells were also included to determine the background absorbance [mean+ (2 x standard deviation)].

2.6.2 Quantitative detection of anti-DHBs antibodies by ELISA

Levels of serum anti-DHBs antibodies were measured using an ELISA described by Miller et al., 2004. Costar® 3590 flat bottom 96-well micro-titre plates were coated with 100 µL per well of a 1/5000 dilution of anti-DHBV pre-S/S mABs, 1H.1 (Pugh et al., 1995) diluted in 0.1 M NaHCO₃ buffer pH 9.6 and incubated at 37°C for 1 hr followed by 4°C O/N. Plates were washed 3 times with PBS-T and were blocked with 200 µL of 5% skim milk in PBS-T per well at 37°C for 1 hr to block any non-specific binding sites. After washing 3 times with PBS-T, 100 µL per well of a 1/500 dilution of sucrose purified DHBsAg (preparation as described by Miller et al., 2004) in 0.05% PBS-T was added to each well at 37°C for 1 hr. Plates were then washed 3 times with PBS-T. Test serum samples were then added in duplicate at a dilution of 1/100 in 5% skim milk
in PBS-T with 4 x five-fold dilutions across the plate. 100 µL per well of a 1/1000 dilution of duck serum sample 262 (a positive control sample containing anti-DHBs and anti-DHBc antibodies) and NDS (negative control) diluted in 5% skim milk in PBS-T were also added to separate wells of the plate with 4 x five-fold dilutions across the plate. Plates were incubated at 37°C for 1 hr followed by 3 washes with PBS-T. Next, 100 µL of a 1/15000 dilution of polyclonal rabbit anti-duck IgY antibodies (Bertram 1997) in 5% skim milk in PBS-T was added to each well and incubated at 37°C for 1 hr. After washing 3 times with PBS-T, 100 µL per well of 1/4000 dilution of HRP-conjugated goat anti-rabbit (Kierkegaard Perry Laboratories Inc. Maryland, U.S.A) diluted in 5% skim milk in PBS-T was added and incubated at 37°C for 1 hr. After 3 washes with PBS plates were developed with OPD as previously described in Section 2.3.1.3.

2.6.3 Detection of anti-DHBc antibodies by ELISA

Levels of serum anti-DHBc antibodies were measured using an ELISA described by Miller et al, 2004. Costar® 3590 flat bottom 96-well micro-titre plates were coated with 100 µL per well of 10 µg/mL purified recombinant DHBcAg (rDHBcAg) in 0.1 M NaHCO₃ buffer pH 9.6 and incubated at 37°C for 1 hr followed by 4°C O/N. Plates were washed 3 times with PBS-T and blocked with 200 µL of 5% skim milk in PBS-T per well at 37°C for 1 hr to block any non-specific binding sites. After washing 3 times with PBS-T, plates were added with 100 µL per well of 1/1000 of test serum samples in 5% skim milk in PBS-T then 4 x five-fold dilution across the plate. Serum from duck 262 and NDS were again used as positive and negative controls and all steps were performed as previously described in Section 2.6.2.

2.7 Histology and immuno-staining of tissue sections
2.7.1 Immuno-staining to detect DHBsAg

Sections of EAA-fixed tissue were incubated at 37°C O/N before being de-waxed by immersing the slides for 2x10 min in AR grade xylene (Merck, Australia). The slides were then washed 2x5 min in AR grade absolute ethanol and rehydrated by 2x5 min washes in PBS. Tissue peroxidase was inactivated by immersing the slides for 1x15 min in 0.5% H2O2 (BDH Chemicals, Australia) in PBS at RT followed by 2x5 min washes in PBS. To minimize non-specific binding, 500 µL of 1/30 dilution of normal sheep serum (NSS) in PBS were placed on each slide and the slides were incubated in a humid box at 37°C, for 30 min. NSS was tipped off onto a paper towel and then 100 µL of a 1/750 dilution of the primary anti-DHBV pre-S/S mAbs, 1H.1 (Pugh et al., 1995) in 10% fetal calf serum (FCS) and 10% NDS in PBS, was placed on each slide with cover slip on and incubated at 37°C for 1 hr then at 4°C, O/N. The next day, slides were dip washed in PBS to remove the cover slips followed by 2x5 min washes in PBS. Slides were then incubated with 200 µL of a 1/250 dilution of HRP-conjugated sheep anti-mouse polyclonal antibodies (Cat. # NA9310V, GE Healthcare Limited, UK) in 10% NDS in PBS, at 37°C for 1 hr, covered with cover-slips and placed in a humid box. After the slides were washed twice in PBS for 5 min, 0.05% of di-amino-benzidine tetrahydrochloride (DAB) (Cat. # D09105, Sigma) and H2O2 (0.04% final) in PBS was distributed over the slides which were kept in the dark for 9 min at RT. The slides were then washed 2x5 min in PBS, and counterstained in Mayer’s haematoxylin for 2 min. Slides were then washed 3x1 min in PBS, and dehydrated for 2x2 min in AR grade 100% ethanol. Finally, slides were immersed for 2x5 min in AR grade xylene and cover slips were mounted on the slides using Depex (Cat. # 61254D, BDH Australia Ltd.,) and the slides were dried in a fume hood O/N. All primary and secondary antibodies used for immuno-staining to detect DHBsAg are listed in Table 2-7.
Slides were examined at 200 x magnification using an eyepiece graticule with 250 x 250 μm grid, and it is expected at least 1x10^5 hepatocytes counted per liver tissue section. The DHBsAg-positive hepatocytes will be stained brown in the hepatocyte cytoplasm and the haematoxylin counter-stained hepatocyte nuclei will be stained blue/purple. To determine the percentage of DHBsAg-positive hepatocytes per 1 x10^5 hepatocytes in each liver tissue section, the number of DHBsAg-positive hepatocytes were divided by the total number of hepatocyte nuclei in the same area and multiplied by 100. The minimum sensitivity of detection of DHBsAg-positive hepatocytes in liver tissues was <0.001%.

2.7.2 Immuno-staining to detect DHBcAg

2.7.2.1 Microwave mediated antigen retrieval
Formalin- or EAA-fixed sections of duck liver were incubated at 37°C O/N before being dewaxed in xylene and rehydrated through ethanol to PBS as described above in Section 2.7.2. For antigen retrieval, the slides were placed in a pot with 250 mL of 10 mM sodium citrate pH 6. The slides were then heated in a microwave on high setting for 3.5 min until boiling and then on a low setting for 10 min. The sections were then cooled in the citrate solution to below 40°C before use.

2.7.2.2 Immuno-staining detection of DHBcAg in liver tissue sections
After antigen retrieval, the slides were washed 2x5 min in PBS followed by immersing the slides in the mixture of H_2O_2 in PBS for 15 min at RT to inactivate tissue peroxidases. The slides were washed 2x5 min in PBS followed by incubation with 200 μL of 10% NDS + 10% FCS + 10% Normal Goat Serum (NGS) in PBS at 37°C, for 30 min, in a humid box, to minimize non-specific binding. The serum was then tipped off onto paper towel and replaced with 100 μL of a 1/400 dilution of anti-DHBc polyclonal antibodies (CQT-2) in 10% NDS +
Table 2-7: Details of antibodies used in immuno-staining to detect specific antigens

<table>
<thead>
<tr>
<th>Specific antigen</th>
<th>Primary antibodies</th>
<th>Secondary antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Description</td>
</tr>
<tr>
<td>DHBpre-S/S</td>
<td>1H.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mouse anti-DHBpre-S/S monoclonal antibodies</td>
</tr>
<tr>
<td>DHBcAg</td>
<td>CQT-1, CQT-2</td>
<td>Rabbit anti-DHBcAg polyclonal antibodies</td>
</tr>
</tbody>
</table>

Note: To detect DHBpre-S/S, primary antibodies were diluted in 10% FCS and 10% NDS in PBS and secondary antibodies were diluted in 10% NDS in PBS.

To detect DHBcAg, all primary and secondary antibodies were diluted in 10% NDS + 10% FCS + 10% NGS in PBS.

Immuno-staining was performed as described in Section 2.7.

References
<sup>a</sup>(Pugh <i>et al.</i>, 1995)
10% FCS + 10% NGS in PBS and incubated at 37°C for 1 hr in humid box then at 4°C, O/N. The next day, slides were dip washed in PBS to remove cover slips followed by washing in PBS for 5 min, twice. Slides were then incubated with 200 μL of a 1/200 dilution of HRP-conjugated Goat anti-rabbit polyclonal antibodies (Cat. # 176-1506, KPL) in 10% NDS + 10% FCS + 10% NGS in PBS, at 37°C for 1 hr, covered with cover-slips and placed in a humid box. All subsequent steps from dip-washing in PBS until mounting the slides in Depex were the same as previously described in Section 2.7.1. All primary and secondary antibodies used for immuno-staining to detect DHBcAg are listed in Table 2-7.

2.8 Methods for extracting and detecting viral and cellular DNA

2.8.1 DNA extraction from duck serum for qPCR
DHBV DNA was isolated and purified from duck serum samples using a Charge Switch® viral nucleic acid extraction kit (Cat. # CS11040, Invitrogen) according to the instructions of the manufacturer. In brief, 200 μL of each duck serum sample was mixed with Charge Switch® Lysis Buffer (140 μL) and Proteinase K (30 μL) and incubated for 20 min at RT. Purification buffer (50 μl) was added and mixed with each sample and 6 μL of Charge Switch® Magnetic Beads were added to bind the DNA at RT, for 2 min or until tight pellets were formed on Magna Rack™. The DNA was washed twice in washing buffer and the DNA was eluted from the filter column in 50 μL of elution buffer. Samples were kept at -20°C before using in qPCR.

2.8.2 DNA extraction from liver tissues for qPCR
Frozen liver tissues collected as described in Sections 2.5.5 and 2.5.6 were thawed and ~25 mg of liver tissues were chopped to a fine paste using a sterile scalpel blade in a plastic petri dish. Total cellular DNA from liver tissues were extracted using DNeasy® Blood and tissue kit (Cat. # 69504, Qiagen) according to the instructions of the manufacturer. In brief, liver
tissue samples were lysed with buffer ATL and proteinase K at 56°C, O/N. The next day, 4 μL of RNase A (100 mg/ml, Cat. # 19191, QIAGEN) was added followed by incubating at RT for 5 min. The DNA samples were then transferred to the DNeasy mini column followed by a series of washing steps. The bound DNA was eluted from the DNeasy mini column in 200 μL of elution buffer. The concentration and quality of samples were determined by Nanodrop1000 spectrophotometer and the samples were stored at -20°C before use in qPCR.

2.8.3 Quantitation of DNA by quantitative PCR

Quantitative PCR were performed in duplicates in MicroAmp Optical 8-tube strips (Applied Biosystems (AB), USA, catalogue no. 4316567 or 4358293) capped with optical caps (AB, USA). Each qPCR master mix consisted of 150 ng of the DNA template, 1X SYBR Green PCR Master Mix (Cat. # 4309155, Applied Biosystems), PCR DW and 6 μM of each forward and reverse DHBV-specific primers (See Table 2-6). The qPCR was then performed using an ABI prism 7000 or AB StepOnePlus™ Real Time PCR machine (Applied Biosystems). In both machines, the same qPCR conditions were used and the samples were subjected to 50°C for 2 min (denaturation step), 95°C for 10 min (to activate the TAQ polymerase). The amplification process then occurred with 40 cycles of 95°C for 15 sec and extension step at 60°C for 1 min. An additional 40 cycles of 60°C to 95°C were performed to determine the dissociation temperature and melt curve for each PCR product. This additional step is to determine whether non-specific amplification or more than one PCR product was present in the reaction.

Standard curves for quantification of DHBV DNA were prepared using plasmid DNA pBL4.8x2 that contains a head-to-tail dimer of the full-length genome of the Australian DHBV (AusDHBV) strain (Triyatni et al., 2001). To construct a standard curve, ~2 μg of plasmid DNA pBL4.8x2 was digested using 20 units of EcoRI (NEB, USA) overnight as
described in Section 2.4.4. The concentration of digested DNA was determined by measuring absorbance at 260 nm using a Nanodrop1000 spectrophotometer (Thermo scientific). Serial dilution of $10^1$ to $10^8$ copies of plasmid DNA were then used to generate the standard curve. To standardise the total amount of DNA present in each qPCR reaction, 150 ng/µL of NDL DNA extracted from NDL samples using the DNeasy blood and tissue kit as described in Section 2.8.2, were also added to each qPCR reaction.

The numbers of copies of target DNA in each sample were quantified through comparison of their cycle threshold ($C_T$) value to the standard curve. The standard curve was generated using a serial diluted of plasmid DNA pBL4.8x2 as template ranged from $10^1$ to $10^8$ copies. All data was analysed using either ABI Prism or StepOne Plus software supplied by AB. The limit of sensitivity of this assay was $10^1$ copies of target DNA.

2.8.4 Detection of DHBV DNA in serum by qPCR

The DHBV DNA levels in the serum were measured by qPCR using primer set 423-576c (See Table 2-6). The DNA template was extracted from serum fraction as described in Section 2.8.1 and was diluted 1/50 in DW. A 5 µL sample of each diluted DNA sample was then added to each master mix as described in Section 2.8.3 and qPCR performed as described in Section 2.8.3.

2.8.5 Detection of DHBV DNA in liver tissue by qPCR

The levels of DHBV total DNA and cccDNA in the liver were quantitative by qPCR using the primer sets 423-576c and CC2–MG1 respectively (See Table 2-6).

Prior to the detection of DHBV DNA by qPCR, extracts of liver were first prepared as described in Section 2.8.2 and then digested with 20 units of EcoRI overnight as described in Section 2.4.4. A 150 ng of the digested liver DNA sample was then added to each master mix
as described in Section 2.8.3 and qPCR performed as described in Section 2.8.3. Each duck cell was reported to contain ~2.5 pg of DNA (Krishan et al., 2005). Therefore, it is expected 150 ng of DNA would contain ~53571 cells equivalent of DNA.

The only difference between the detection of DHBV DNA and cccDNA in liver tissues by qPCR was the specific primers used in each reaction which was stated in Table 2-6. The primer set (423-576c) was designed to bind sequences of the polymerase ORF of the DHBV genome. This allows the DHBV total DNA present in the DNA sample can be amplified by primers 423–576c as this region is intact in all forms of DHBV DNA (DHBV rcDNA, RI and cccDNA) (Reaiche et al., 2010).

On the other hand, the cccDNA primer set (CC2-MG1) binds specifically to the cohesive overlap region of the DHBV rcDNA (Figure 2.1). When the double-stranded cccDNA is denatured, two complete circular strands can be amplified by the CC2-MG1 primer set. In contrast, the rcDNA is incomplete at 3’ positive strand and has a gap in the negative strand DNA. Therefore, when the rcDNA is denature, the two linear strand of DNA could not be amplified by CC2-MG1 primer set (Reaiche et al., 2010).

2.9 Statistical analysis

To determine the statistical significance of the difference in data obtained from different groups of ducks, data was analysed using repeated-measures ANOVA (PROC GLM) with time as the factor. Pair wise post-hoc comparison between treatments without correction was conducted. Significance was assessed at the 5% level. All analyses were performed using SAS v9.3 (SAS Institute, Cary, NC, USA).
Figure 2.1: Schematic representation of the AusDHBV genome (GenBank Accession AJ006350) in its rcDNA form. The approximate binding sites of PCR primers sets to amplify DHBV total (423 – 576c) and cccDNA (MG1-CC2) are shown in the diagram and the primer sequences are shown in Table 2-6.

Symbols “+” and “-” represent the DHBV rcDNA positive and negative DNA strands respectively.

(Adapted with permission from Huey Low Ph.D. Thesis, The University of Adelaide, 2012)
Chapter 3: Production of polyclonal and monoclonal antibodies specific for DHBcAg

3.1 Background

Studies of HBV-infected liver tissue to determine the percentage infected hepatocytes have focused on detection of HBsAg in the hepatocyte cytoplasm and HBeAg in the cytoplasm and nucleus of hepatocytes (Gowans and Burrell, 1985; Kakumu et al., 1989). In a similar way, studies in DHBV-infected ducks have focused on the two structural proteins of DHBV, DHBsAg and DHBcAg. Currently, immuno-staining of DHBsAg with monoclonal anti-pre-S/S antibodies (1H.1) (Pugh et al., 1995) is used routinely in our laboratory to determine the percentage of DHBsAg-infected hepatocytes in EAA-fixed liver tissue sections. This assay allows detection of as few as 0.001% of infected hepatocytes with low levels of background staining (Foster et al., 2003, 2005; Miller et al., 2006, 2008; Feng et al., 2010).

Immuno-staining techniques for the detection of hepadnavirus core antigens have been used to identify infected WHV–infected hepatocytes in the woodchuck model (Mason et al., 2004) and HBV-infected hepatocytes in the chimpanzee model (Mason et al., 2009). In contrast, detection of DHBcAg-positive hepatocytes is not commonly used in the duck model. Currently, only limited supplies of polyclonal rabbit anti-DHBc antibodies (Rabbit R39408; Jilbert et al., 1992) are available in our laboratory. In addition, previous attempts in our laboratory to produce mABs by immunising mice with purified rDHBcAg protein or with DHBcAg expressed from DNA vaccines had been unsuccessful (Stephen Blake Honours Thesis, 2004; Darren Miller, personal communication). Therefore, the first aim of this Ph.D. project was to produce specific polyclonal and mABs against DHBcAg.

This Chapter includes a description of: (i) the purification and preparation of rDHBcAg proteins used for the immunisation of rabbits and mice; (ii) the production of polyclonal and
mABs against DHBcAg and (iii) the development and optimisation of immuno-staining techniques for the detection of DHBcAg which included immuno-staining, Western blot and IMF assays.

Immuno-staining of DHBcAg in duck liver sections is an essential part of this Ph.D. project as it will allow comparison with the percentage of DHBsAg-positive hepatocytes to more accurately determine the extent of DHBV infection in the liver. The detection of DHBcAg in the liver of DHBV-infected ducks was then used in the studies described in Chapters 5 and 6, to evaluate the efficacy of our vaccination strategies against DHBV infection.

### 3.2 Purification of rDHBcAg particles expressed in E. coli

The rDHBcAg proteins used in this study were expressed intracellularly in E. coli where they self-assemble into nucleocapsid particles (Birnbaum and Nassal, 1990; Kann and Gerlich, 1994) comprised of 180-240 subunits of rDHBcAg protein (Hui et al., 1999). The nucleocapsid particles comprised of rDHBcAg were harvested and then purified by CsCl density gradient isopycnic centrifugation as described below.

#### 3.2.1 Preparation and purification of rDHBcAg

To prepare rDHBcAg, the DHBcAg gene (nucleotides 2647-677, of the USA strain of DHBV, DHBV16; Mandart et al., 1984) was cloned downstream of an IPTG inducible promoter into a plasmid (pKK223amp) to create pKK223amp-DHBc as described in Section 2.3.1.1. Subsequently, an E. coli strain JM101 was transformed with pKK223amp-DHBc to create a recombinant bacterial strain that was grown in bacterial culture and induced by IPTG to produce significant amounts of rDHBcAg as described in Section 2.3.1.1 and as previously published by (Jilbert et al., 1992). To verify the presence of the DHBcAg gene in the recombinant E. coli strain, PCR was performed using specific primers as listed in Table 2-6. As shown in Figure 3.1, a 500 bp PCR product was observed in Lane 3 (JM101 containing
Figure 3.1. Verification of the rDHBcAg gene in pKK223amp-DHBc using PCR.

The rDHBcAg gene in the *E. coli* strain JM101, transformed with pKK233amp-DHBc, was amplified with a set of primers, 5’TAGAGCCTTAGCCAATGTG3’, and 5’GGAGGGTTACAACCAAGTACG3’. The PCR products were analysed on a 1.2% agarose gel, and the amplified DNA band was of the expected size of ~508 bp (Lane 3). No band was observed in Lane 2 (JM101 transformed with pKK233amp), and Lane 4 (water control). Lane 1 is the GeneRuler™ 100 bp DNA Marker (Thermo Scientific) molecular weight marker.

1. GeneRuler™ 100 bp DNA Marker
2. Resuspended JM101 transformed with pKK233amp
3. Resuspended JM101 transformed with pKK233amp-DHBc
4. Water negative control
pKK233amp-DHBc), but not in Lane 2 (JM101 containing pKK233amp) or in Lane 4 (water control). Since the predicted PCR product from the amplified DHBcAg gene was 508 bp, an absence of PCR product in the negative controls (Lanes 2 and 4) confirmed that the PCR product in Lane 3 was due to the presence of the cloned DHBcAg gene.

To collect the rDHBcAg particles, the IPTG-induced bacterial cells were harvested and lysed using a French press, twice, to release rDHBcAg particles into the supernatant. The cell lysate was separated from cell debris by centrifugation and the supernatant was collected. The rDHBcAg particles in the supernatant were then precipitated using ammonium sulphate [(NH₄)₂SO₄], at a final concentration of 33.5%, in an ice bath for 1 hr. The precipitated rDHBcAg particles were recovered by centrifugation and dissolved in 20 mM PB (pH 6.8) followed by dialysis against the same buffer O/N. The (NH₄)₂SO₄ and other contaminants were removed by centrifugation and the precipitation and dialysis process was repeated five times as described in Section 2.3.1.1. After the fifth precipitation and dialysis process, the supernatant contained semi-purified rDHBcAg particles.

3.2.2 Separation and isolation of rDHBcAg particles by CsCl density gradient centrifugation

The semi-purified rDHBcAg particles were further purified by isopycnic centrifugation on CsCl density gradients as described in Section 2.3.1.2. The supernatants containing the rDHBcAg particles were layered on top of a CsCl gradient consisting of 1.2, 1.5 and 1.7 gm CsCl/cm³ in Tris-EDTA in a SW41 centrifuge tube as described in Section 2.3.1.2. After centrifugation at 210,000 g, at RT for 20 hr, 1-2 white bands were visible in the CsCl gradient. 0.5 ml fractions were collected from the bottom of the tube and subjected to ELISA testing for the presence of rDHBcAg particles.
3.2.3 Detection of rDHBcAg particles by ELISA

ELISA assay was performed as described in Section 2.3.1.3 to determine which fractions from the CsCl gradient contained the highest amount of rDHBcAg particles. Since the white bands were visible in fractions 9 to 12, aliquots taken from fractions 7-12 were tested in ELISA. As shown in Figure 3.2, ELISA intensities were constantly low in all tested fractions when probed with NDS. The observed low background reading could be due to the presence of anti-\textit{E. coli} antibodies in NDS that detect traces of \textit{E. coli} protein in the rDHBcAg samples in each fraction. In contrast, when probed with duck anti-DHBcAg polyclonal antibodies (from duck 262), fractions 9-12 were higher in ELISA intensities. In addition, the CsCl density of the pooled fractions 9-12 was 1.336 g/cm$^3$ (data not shown), which was close to the density of rDHBcAg particles that is reported to be 1.36-1.42 gm CsCl/cm$^3$. This indicated that the rDHBcAg particles were found in fractions 9-12.

Since the presence of CsCl in the protein samples will affect protein migration, aliquots of fractions 9-12 were subjected to a TCA precipitation process as described in Section 2.3.1.4 to remove CsCl from the collected fractions prior to SDS-PAGE.

3.2.4 SDS-PAGE and Western Blot analysis of the purity of rDHBcAg particles

Figure 3.3 is a Coomassie blue stained SDS-PAGE showing the purity of the rDHBcAg particles before (Lane 1) and after CsCl density gradient centrifugation (Lanes 2-6). As can be seen, other than fraction 8 (Lane 2), 1 band at size $\sim$33 kDa that corresponds to the rDHBcAg protein was detected in all tested samples with low levels of contaminants, showing no difference in the purity of rDHBcAg particles. This indicated that precipitation with 33.5\% (NH$_4$)$_2$SO$_4$ followed by CsCl density gradient centrifugation efficiently purified the rDHBcAg particles while leaving most of the contaminants in the solution.
In Figure 3.4, the same protein samples were loaded in the gel and rDHBcAg proteins were identified by Western Blot using polyclonal rabbit anti-rDHBc antibodies (R39408) as described in Section 2.2.7. There were more bands in Western Blot compared to the Coomassie blue stained gel, indicating that Coomassie blue staining was less sensitive in detecting proteins compared to Western Blot that utilised antibodies specific for the rDHBcAg.

In the Western Blot assay, 2 major bands were detected in Lanes 1, 3, 4, 5, and 6, which were ~33 kDa and ~64 kDa in size. As mentioned in Section 1.2.2, in human HBV, HBcAg and HBeAg (the secreted form of HBcAg) are translated from the C-ORF. HBcAg can be detected in the liver tissue of patients with CHB and it is known that the assembly of nucleocapsid requires the dimerisation of the HBcAg protein (Kann, 2008; Damme et al., 2013). The HBeAg is not part of the virus particle but is a secreted protein that can be detected in the serum of patients with CHB (Seeger and Mason, 2000; Bruss, 2004; Damme et al., 2013). In this study, the cloned rDHBcAg gene was 789 bp, corresponding to a coding capacity of 263 amino acids. Taking that the average molecular weight of an amino acid is 136 Da, the rDHBcAg protein band in Western Blot is estimated to be ~36 kDa (263 x 136 Da = 36000 Da).

Therefore, in Figure 3.4, the ~33 kDa protein corresponds to the rDHBcAg protein and by analogy; the protein at ~64 kDa could be a dimer of rDHBcAg protein. Another band at ~20 kDa was also detected in Lanes 1, 3 and 4, which was thought to be due to the breakdown of rDHBcAg proteins. In consistent to the Coomassie blue stained gel (Figure 3.3), Fraction 8 showed no band in Western Blot (Figure 3.4; Lane 2), indicating that the rDHBcAg particles were lost during the TCA precipitation process.
Figure 3.2. ELISA reaction intensity (A$_{490\text{ nm}}$) of rDHBcAg particles in each fraction, probed with either duck anti-DHBcAg polyclonal antibodies (duck 262) or NDS.

An ELISA assay was performed to determine which fractions of the CsCl gradient contained the highest amount of rDHBcAg particles. The results showed that Fractions 9-12 were higher in ELISA intensities when probed with duck anti-DHBcAg polyclonal antibodies from duck 262 (1/5,000 dilution); indicating the presence of rDHBcAg particles in these fractions. For a negative control, ELISA intensities were consistently low in all tested fractions probed with NDS (1/5,000 dilution).
**Figure 3.3. 12% SDS-PAGE showing the purity of the rDHBcAg preparation.**

After the rDHBcAg proteins were precipitated using TCA, 20 μL of each denatured protein sample was loaded onto each lane of SDS-PAGE gel. The gel was electrophoresed, stained with Coomassie blue and lastly washed in de-staining solution before visualisation on a light box as described in Section 2.2.7. The results revealed a ~33 kDa band of rDHBcAg in all tested samples with low levels of contaminants, showing no difference in purity of the crude rDHBcAg proteins before (Lane 1) and after CsCl density gradient centrifugation (Lanes 2-6).

Lane 1: Crude rDHBcAg protein sample
Lane 2: Purified rDHBcAg particles in Fraction 8
Lane 3: Purified rDHBcAg particles in Fraction 9
Lane 4: Purified rDHBcAg particles in Fraction 10
Lane 5: Purified rDHBcAg particles in Fraction 11
Lane 6: Purified rDHBcAg particles in Fraction 12
Figure 3.4. Western blot showing the rDHBcAg preparation to be used in the immunisation of rabbits and mice.

The same protein samples that were used in Coomassie blue staining were loaded onto another gel and rDHBcAg proteins were identified by Western Blot using polyclonal rabbit anti-rDHBc antibodies (R39408). Two major bands were detected in Lanes 1, 3, 4, 5, and 6, which were ~33 kDa and ~64 kDa in size. The ~33 kDa protein corresponds to the rDHBcAg protein and the protein at ~64 kDa was thought to be dimers of rDHBcAg. Another band at ~20 kDa was also detected in Lanes 1, 3 and 4, which could be the breakdown of rDHBcAg proteins. There were multiple bands in different sizes observed in Lane 1 but not in other Lanes. This indicated the presence of contaminating proteins in the crude rDHBcAg preparation from the *E. coli* lysate. Consistent with Coomassie blue stained gel (Figure 3.3), Fraction 8 showed no band in Western Blot (Lane 2).

<table>
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<tr>
<th>Lane</th>
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Lane 1: Crude rDHBcAg protein sample  
Lane 2: Purified rDHBcAg particles in Fraction 8  
Lane 3: Purified rDHBcAg particles in Fraction 9  
Lane 4: Purified rDHBcAg particles in Fraction 10  
Lane 5: Purified rDHBcAg particles in Fraction 11  
Lane 6: Purified rDHBcAg particles in Fraction 12
Apart from the three major bands mentioned above, multiple bands of different sizes were observed in the sample before CsCl centrifugation (Lane 1) but not in other lanes. Since the polyclonal antibodies R39408 were produced in a rabbit immunised with rDHBcAg proteins extracted from *E. coli*, the contaminants in Lane 1 (Figure 3.4) could be the *E. coli* protein reacted with anti-*E. coli* antibodies produced in rabbit R39408. This indicated that many contaminating proteins were present in the semi-purified rDHBcAg particle preparation derived from *E. coli* lysates, suggesting that CsCl density gradients centrifugation efficiently separated and purified the rDHBcAg particles from the *E. coli* lysates, and removed impurities that were still present after the precipitation with (NH₄)₂SO₄. This was expected as CsCl density gradient centrifugation has been shown to be a powerful tool to purify other viruses such as adenovirus (Arándiz-Borunda *et al.*, 2011; Mueller *et al.*, 2012). In this study, CsCl density gradient centrifugation separated the rDHBcAg particles based on their specific density, removing contaminants with a different density that became trapped within other CsCl layers. Therefore, further purification of the semi-purified rDHBcAg protein samples by using CsCl density gradient centrifugation was necessary.

To immunise rabbits and mice for production of polyclonal and mABs, fractions containing the rDHBcAg particles were pooled, and concentrated using an Amicon Ultra-15 centrifugal filter. The final protein concentration was determined using a Quick Start™ Bradford protein assay as described in Section 2.3.1.5. The final yield of purified rDHBcAg was ~6 mg/liter of the original *E. coli* culture.

**3.3 Production of polyclonal anti-DHBc antibodies.**

**3.3.1 Immunisation of rabbits to generate polyclonal anti-DHBc antibodies.**

Two rabbits (CQT-1 and CQT-2) were immunised with purified rDHBcAg (500 μg per dose) combined with either FCA or FIA, 4 times over a 6-week period as described in Section 2.3.2.
It was previously shown that the emulsification of antigens in Freund’s adjuvant had minimum effect on conformational change of the protein antigens and the adjuvant enhancement of the immunogenicity is not through conformational change of the protein antigens (Berzofsky et al., 1976). Thus, it was believed that the rabbits were immunised with immunogens that have same confirmation as the DHBV nucleocapsid. The blood samples were taken from each rabbit before immunisation, and 10 days after the third dose to prepare serum samples that were tested for anti-core antibodies by ELISA as described in Section 2.3.2.1. As shown in Figure 3.5, the pre-bleed samples from both rabbits had low levels of anti-\textit{E. coli} antibodies that detect traces of \textit{E. coli} protein in rDHBcAg that was used to coat the ELISA plates, resulting in the observed background reading. By comparing the pre-bleed samples and test-bleed samples from both rabbits, specific antibody response was also observed in these two rabbits after immunisation. As can be seen in Figure 3.5, strong specific antibody responses were induced in both rabbits following 3 doses of immunisation and anti-DHBc antibodies were titratable to 1/390625. The results also showed that specific antibody responses in rabbits CQT-1 and CQT-2 were similar to the positive control, R39408 (polyclonal rabbit anti-DHBc antibodies). Therefore, 10-14 days after the fourth dose was given to the rabbits, exsanguination was performed. Blood samples were collected and approximately 60 mL of serum was produced from each rabbit.

3.3.2 Optimisation of the use of polyclonal anti-DHBc antibodies (CQT-1 and CQT-2).

3.3.2.1 IMF staining of DHBcAg in tet-induced dstet5 cells using polyclonal anti-DHBc antibodies (CQT-1 and CQT-2).
To confirm whether the antisera bound to DHBcAg, an IMF assay using the cell line, dstet5, was performed as described in Section 2.2.6. The dstet5 cell line is a stably transfected sub-line of the chicken hepatoma LMH cell line that expresses a DHBV strain, DHBV1S, under the control of the tetracycline (tet)-regulated promoter (Guo et al., 2003). DHBV1S is an
envelope-deficient DHBV mutant and, as a consequence, infection of cells with this mutant results in the intracellular accumulation of mature nucleocapsids that cannot be secreted from the cells. R39408 and NRS were used as positive and negative controls respectively. Figure 3.6 showed that specific cytoplasmic IMF staining was observed in tet-induced dstet5 cells when CQT-1, CQT-2 and R39408 were used as the primary antibodies. DHBcAg is traditionally detected throughout the whole cytoplasm of cells and not in the nucleus (Jilbert et al., 1992). No IMF staining was seen in tet-induced dstet5 cells incubated with NRS. In comparison to R39408 that was used at a dilution of 1/200, both CQT-1 and CQT-2 were able to be used in lower concentration (1/700) with a 1/200 dilution of secondary antibodies (Table 2-2), and only low levels of background IMF staining were present.

3.3.2.2 Western Blot analysis using polyclonal anti-DHBc antibodies (CQT-1 and CQT-2).

The proteins present in lysates of LMH and tet-induced dstet5 cells were extracted and used in Western Blot assays as described in Section 2.2.7. The tet-induced dstet5 cells have a surface antigen mutation that inhibits virus secretion and they accumulate high numbers of DHBV nucleocapsid particles which was used to facilitate optimisation of this assay. Four concentrations of CQT-1 and CQT-2; 1/500, 1/2500, 1/12500 and 1/62500 were used. The optimum dilution of these antisera against DHBcAg was between 1/12500 and 1/62500 with a 1/50,000 dilution of secondary antibodies (Table 2-4). As can be seen in Figure 3.7, with CQT-1 and CQT-2, immunblotting revealed a sharp band at the position of ~33 kDa with protein extracted from tet-induced dstet5 cells, corresponding to the DHBcAg (Lanes 1, 3, 5 and 7). This band was not observed in protein extracted from LMH cells probed with same antiserum at the same concentration (Lanes 2, 4, 6 and 8). The results also demonstrated that CQT-2 gave stronger signals compared to CQT-1 at the same concentration, suggesting CQT-2 was more sensitive in Western Blot. The bottom panel of Figure 3.7 showed that the intensity of β-actin specific bands was similar in all tested samples suggesting that similar
Figure 3.5 ELISA assay to determine the titre of anti-DHBc antibodies in the test bleed of rabbits after immunisation with the 3rd dose of purified rDHBcAg protein.

Two rabbits (CQT-1 and CQT-2) were injected s.c. with purified antigen (500 µg per dose) 4 times over a 6-week period as described in Section 2.3.2. Serum samples were taken from each rabbit before immunisation and 10 days after the 3rd dose to test the antibody titre by ELISA. Results demonstrated a specific antibody response in these two rabbits was absent before immunisation and strong specific antibody responses were induced in both rabbits following 3 doses of rDHBcAg. The results also showed that specific antibody responses in both rabbits CQT-1 and CQT-2 were similar to R39408. Both rabbits CQT-1 and CQT-2 were given a 4th dose of rDHBcAg and were exsanguinated 10-14 days later. ~60 mL of serum was collected from each rabbit.
Figure 3.6. IMF detection of DHBcAg in tet-induced dstet5 cells using polyclonal anti-DHBc antibodies.

The dstet5 cells were cultured in GI medium 1640 supplemented with phenol red as described in Section 2.2.4 and were fixed with pre-chilled 95:5 EAA as described in Section 2.2.6. Specific IMF in tet-induced dstet5 cells was observed when CQT-1, CQT-2 and R39408 were used as primary antibodies. No IMF was seen in tet-induced dstet5 cells incubated with NRS.

Panels (i) tet-induced dstet5 cells counter-stained with DAPI to detect cell nuclei, (ii) tet-induced dstet5 cells stained with FITC-labelled antibodies to detect the cytoplasmic DHBcAg expression and (iii) A merged image of tet-induced dstet5 cells stained with DAPI and FITC using imaging software. All photos were taken at 100 x magnification.

Panel A. CQT-1 primary antibodies, at a 1/700 dilution
Panel B. CQT-2 primary antibodies, at a 1/700 dilution
Panel C. R39408 primary antibodies, at a 1/200 dilution (positive control)
Panel D. NRS primary antibodies, at a 1/200 dilution (negative control)
Figure 3.7. Western blot detection of DHBcAg in tet-induced dstet5 cells using polyclonal anti-DHBc antibodies (CQT-1 or CQT-2).

The protein from LMH and tet-induced dstet5 cells (~3x10^5 cells) were extracted and used in Western Blot assay as described in Section 2.2.7. The optimum concentration of these antisera (CQT-1 or CQT-2) was between dilutions of 1/12500 and 1/62500 with a 1/50000 dilution of secondary antibodies (Table 2-4).

Upper panel: Immunblotting with CQT-1 or CQT-2 revealed a sharp band at the position of ~33 kDa with protein extracted from tet-induced dstet5 cells, corresponding to the DHBcAg (Lanes 1, 3, 5 and 7). This band was not observed in protein extracted from LMH cells probed with the same antiserum at the same concentration (Lanes 2, 4, 6 and 8).

Lower panel: The intensity of β-actin specific bands was similar in all tested samples suggesting that similar amounts of protein were loaded in each lane of the gel.
amounts of protein were loaded in each sample. Duck β-actin was detected using anti-human β-actin antibodies (Feng et al., 2010) used at a dilution of 1/10,000 (Table 2-4).

To confirm that the CQT-1 and CQT-2 antibodies were specific for DHBcAg and do not cross-react with E. coli proteins, a parallel Western Blot was performed on protein extracted from a recombinant E. coli strain JM101 that was transformed with pKK233amp-DHBc and also from E. coli strain JM101 that was transformed with plasmid vector pKK223amp (Figure 3.8). The results showed that with CQT-1 and CQT-2, specific bands at size ~33 kDa were observed in all protein samples extracted from JM101 with pKK233amp-DHBc, corresponding to the DHBcAg (Lanes 1, 3, 5 and 7). No band was observed in protein samples extracted from JM101 transformed with plasmid vector pKK233amp (Lanes 2, 4, 6 and 8). These results indicated that the CQT-1 and CQT-2 antibodies did not bind to E. coli proteins and were specific for core antigens. Nonetheless, the specific band at Lane 3 detected with CQT-1 at 1/62500 dilution was relatively faint compared to bands in Lane 1, 5 and 7. This finding was again confirmed that CQT-2 was more sensitive than CQT-1 in Western Blot. The lower panel of Figure 3.8 showed protein loading control using anti-Beta-lactamase antibodies as described in Table 2-4. These beta-lactamase antibodies were raised to detect beta-lactamase derived from E. coli (Millipore, data sheet 2013).

To further confirm the specificity of CQT-1 and CQT-2, Western Blot was performed on proteins extracted from duck liver tissue (Figure 3.9). 50 mg of liver tissue from a persistently DHBV-infected duck and non-DHBV-infected duck was homogenised separately in lysis buffer (Table 2-3). The protein in the homogenate supernatant was then collected, denatured and added with loading buffer before applied to SDS-PAGE and Western Blot as described in Section 2.2.7. A DHBV-infected duck liver extract was also probed with NRS a negative control (Figure 3.9, lane 5).
It is known that the secreted protein, HBeAg, does not form aggregates and is detected in the serum (Seeger and Mason, 2000; Bruss, 2004; Damme et al., 2013). Similarly, DHBcAg (Schlicht et al., 1987) and WHV e antigen (WHeAg) (Carlier et al., 1994) can be detected in the serum as glycosylated proteins. Therefore, DHBcAg and WHeAg were not released from lysed cells but rather are actively secreted. In contrast, HBcAg assembles into nucleocapsid particles and is predominantly detected in the liver (Kann, 2008; Damme et al., 2013). By analogy, in Figure 3.9, the major protein band at the position of ~33 kDa (Lanes 2 and 4), corresponded to liver-derived non-secreted DHBcAg. No band was observed in the homogenate samples from NDL (Lanes 1 and 3) or in a DHBV-infected duck liver homogenate probed with NRS (Lane 5). These data indicated that CQT-1 and CQT-2 were able to detect liver-derived DHBcAg proteins.

3.3.2.3 Detection of DHBcAg-positive hepatocytes in liver tissue sections.

In order to detect DHBcAg-positive hepatocytes in duck liver tissue, immuno-staining techniques with polyclonal anti-DHBc antibodies were developed as described below. Only the results of immuno-staining with CQT-2 were shown here due to its optimum staining compared to CQT-1 (data not shown).

EAA-fixed (slides # 4869 and 4871) and formalin-fixed (slide # 4872) liver tissue sections of 6-week-old ducks were used to optimise this staining. The EAA-fixed liver tissue sections showed 2-3% of DHBsAg-infected hepatocytes when probed with monoclonal anti-pre-S/S antibodies (1H.1) (Pugh et al., 1995). A comparison of the staining was made using liver tissue sections from uninfected 6-week-old ducks fixed in EAA (slide # E64388) and formalin (slide # 6122).
Figure 3.8. Western blot analysis using polyclonal anti-DHBc antibodies (CQT-1 or CQT-2) of DHBcAg expression in the *E. coli* strain JM101 transformed with pKK223amp-DHBc.

To confirm that the antiserum (CQT-1 and CQT-2) were specific for DHBcAg and did not cross-react with *E. coli* proteins, proteins extracted from JM101 transformed with pKK233amp-DHBc and JM101 transformed with the vector construct pKK223amp were used in Western Blot assay as described in Section 2.2.7.

Upper panel: Immunoblotting with CQT-1 or CQT-2 revealed a sharp band at the position of ~33 kDa with protein extracted from JM101 transformed with pKK233amp-DHBc, corresponding to the rDHBcAg (Lanes 1, 3, 5 and 7). This band was not observed in protein extracted from the control *E. coli* strain probed with the same antiserum at the same concentration (Lanes 2, 4, 6 and 8).

Lower panel: The intensity of beta-lactamase bands was similar in all tested samples suggesting that similar amounts of protein were loaded in each lane of the gel.
Figure 3.9. Western blot analysis of DHBcAg expression in NDL and DHBV-infected duck liver using polyclonal anti-DHBc antibodies (CQT-1 or CQT-2).

50 mg of DHBV-infected duck liver and 50 mg of non DHBV-infected NDL was homogenised separately in lysis buffer (Table 2-3). The protein in the homogenate supernatant was then collected, denatured and mixed with loading buffer before being analysed in Western Blot as described in Section 2.2.7.

**Upper panel:** Immunoblotting with CQT-1 or CQT-2 revealed a sharp band at the position of ~33 kDa with protein extracted from DHBV-infected duck liver, corresponding to the liver-derived DHBcAg (Lanes 2 and 4). This band was not observed in the homogenate samples from NDL (Lanes 1 and 3) that were probed with same antiserum at same concentration or in a DHBV-infected liver homogenate sample probed with NRS (Lane 5).

**Lower panel:** The intensity of β-actin specific bands was similar in all tested samples suggesting that similar amounts of protein were loaded in each lane of the gel.

Lane 1: NDL  
Lane 2: DHBV-infected duck liver  
Lane 3: NDL  
Lane 4: DHBV-infected duck liver  
Lane 5: DHBV-infected duck liver

- CQT-1 primary antibodies, at a 1/12500 dilution
- CQT-2 primary antibodies, at a 1/12500 dilution
- NRS primary antibodies, at a 1/12500 dilution
In sections of EAA-fixed liver tissue, the primary anti-DHBc antibodies (CQT-2) were used at six different concentrations; 1/50, 1/100, 1/500, 1/1000, 1/1500 or 1/2000 and the concentration of the secondary goat anti-rabbit antibodies was 1/250. The results showed that DHBcAg-positive hepatocytes were detected only when the primary antibodies were used at concentrations of 1/500 or 1/1000. This was not seen when the non-DHBV-infected control liver tissue (slide # E64388) was used (Figure 3.10). The DHBcAg-positive hepatocytes were detected more efficiently in sections of EAA-fixed liver tissue treated with antigen retrieval (slide # 4869; Figure 3.11) than in sections EAA-fixed liver tissue that were not treated for antigen retrieval (slide # 4869; Figure 3.12). In general, the positive staining in EAA-fixed liver sections without antigen retrieval had better cellular morphology but less immuno-staining intensity compared to liver sections with antigen retrieval. However, in antigen retrieval tissues, some shrinkage of cells was observed with less visible nuclei and hence accurate cell counting was not possible. This is expected as antigen retrieval is not recommended following EAA-fixation as it is known to compromise the integrity of the tissue sections or cells (rndsystems, Technical Information, 2013).

To increase the specificity of the staining and to further reduce background staining, CQT-2 was absorbed with NDL and NDS as described in Section 2.3.2.2. In addition, formalin-fixed liver tissue was used to determine if formalin fixation improved the preservation of cell structure after the antigen retrieval process. The primary antibodies (CQT-2 adsorbed with NDL and NDS) were used at seven different concentrations; 1/25, 1/50, 1/100, 1/200, 1/400, 1/500 or 1/1000 and the goat anti-rabbit secondary antibodies were used at three concentrations; 1/150, 1/200 and 1/250. The results showed that DHBcAg-positive hepatocytes were detected more efficiently in sections of formalin-fixed liver tissue (slide # 4872) with antigen retrieval than in sections of EAA-fixed liver tissue (slide # 4871). As can be seen in Figure 3.13, in formalin-fixed tissues with antigen retrieval, the cell structure was preserved and the staining of DHBcAg-positive hepatocytes was strong with low levels of
background staining. The optimum concentration of primary antibodies was 1/400 of CQT-2 adsorbed with NDL and NDS, and with a 1/200 dilution of secondary antibodies as described in Section 2.7.2.2. Both EAA- and formalin-fixed liver tissue showed a strong cytoplasmic staining pattern but not membranous staining pattern in DHBcAg-infected hepatocytes. Nonetheless, superior cell morphology was observed in formalin-fixed liver tissue after the antigen retrieval process, with a sharp outlining of the nucleus and cytoplasm, as compared to EAA-fixed liver tissue.

### 3.3.2.4 Comparison of the sensitivity of detection of DHBsAg- and DHBcAg-positive hepatocytes in liver tissue sections.

Since liver tissue sections in slides # 4871 (EAA-fixed) and 4872 (formalin-fixed) were collected at the same time from the same duck (duck 141), immuno-staining to detect DHBsAg- and DHBcAg-infected hepatocytes was performed to compare the number of DHBV-infected hepatocytes (Figure 3.14). The immuno-staining to detect DHBsAg-infected hepatocytes was performed with monoclonal anti-pre-S/S antibodies (1H.1) (Pugh et al., 1995) in sections of EAA-fixed liver tissue without antigen retrieval (slides # 4871). The immuno-staining to detect DHBcAg-infected hepatocytes was performed with CQT-2 (adsorbed with NDL and NDS) in sections of formalin-fixed liver tissue with antigen retrieval (slides # 4872). The results showed that the percentage of DHBsAg- and DHBcAg infected hepatocytes were 2.2% and 1.9% respectively. From the observation, immuno-staining to detect DHBsAg- and DHBcAg-infected hepatocytes show similar distribution and pattern of staining within the cytoplasm of the cell (Figure 3.14). This finding indicated the sensitivity of immuno-staining to detect DHBsAg- and DHBcAg-infected hepatocytes were similar and therefore DHBV-infected cells in liver tissues could be detected either by DHBsAg or DHBcAg staining.
Figure 3.10. Immuno-staining of EAA-fixed liver section for the detection of DHBcAg-positive hepatocytes.

EAA-fixed liver tissue sections from a non DHBV-infected 6-week-old duck (slide # E64388) stained with polyclonal anti-DHBc antibodies (CQT-2) as described in Section 2.7.2.2., showing no positive hepatocytes (A) at a 1/50 dilution, (B) at a 1/100 dilution.

Panel (i) The liver tissue sections were photographed using 100 x magnification; Bar = 200 µm and (ii) 400 x magnification; Bar = 50 µm.
Figure 3.11. Immuno-staining of EAA-fixed liver section with antigen retrieval for the detection of DHBcAg-positive hepatocytes.

**Panel (A)** EAA-fixed liver tissue sections from a DHBV-infected 6-week-old duck (slide # 4869) with antigen retrieval stained with polyclonal anti-DHBc antibodies (CQT-2) at a 1/500 dilution, and (B) at a 1/1000 dilution as described in Section 2.7.2.2. Antigen retrieval resulted in deterioration of the morphology of the cells making it difficult to determine the percentage of DHBcAg-positive hepatocytes.

**Panel (i)** The liver tissue sections were photographed using 200 x magnification; Bar = 100 µm and (ii) 400 x magnification; Bar = 50 µm.
Figure 3.12. Immuno-staining of EAA-fixed liver sections without antigen retrieval for the detection of DHBcAg-positive hepatocytes.

Panel (A). EAA-fixed liver tissue sections from a 6-week-old DHBV-infected duck (slide # 4869) processed without antigen retrieval stained with polyclonal anti-DHBc antibodies (CQT-2) as described in Section 2.7.2.2., at a 1/50 dilution, showing 1.2% of DHBcAg-positive hepatocytes and (B) at a 1/100 dilution showing 0.73% of DHBcAg-positive hepatocytes.

Panel (i) The liver tissue sections were photographed using 200 x magnification; Bar = 100 µm and (ii) 400 x magnification; Bar = 50 µm.
Figure 3.13. Immuno-staining of a formalin-fixed liver section with antigen retrieval for the detection of DHBcAg-positive hepatocytes.

Panel (A) Formalin-fixed liver tissue sections from a 6-week-old DHBV-infected duck (slide # 4872) with antigen retrieval stained with polyclonal anti-DHBc antibodies (CQT-2) absorbed with NDL and NDS at a 1/400 dilution as described in Section 2.7.2.2, with secondary antibodies (goat anti-rabbit HRP) at a 1/200 dilution, and (B) with secondary antibodies at a 1/250 dilution, showing 1.9% and 1.2% of DHBcAg-positive hepatocytes respectively.

Panel (i) The liver tissue sections were photographed using 200 x magnification; Bar = 100 µm and (ii) 400 x magnification; Bar = 50 µm.
Figure 3.14. Immuno-staining of EAA-fixed liver sections for the detection of DHBsAg-positive hepatocytes and a formalin-fixed liver section with antigen retrieval for the detection of DHBcAg-positive hepatocytes.

Panel (A) EAA-fixed liver tissue sections from a 6-week-old DHBV-infected duck (slide # 4871) stained with a 1/750 dilution of monoclonal anti-pre-S/S antibodies (1H.1) (Pugh *et al.*, 1995), and with a 1/250 dilution of secondary antibodies as described in Section 2.7.1, showing 2.2% of DHBsAg-positive hepatocytes.

Panel (B) Formalin-fixed liver tissue sections from a 6-week-old DHBV-infected duck (slide # 4872) with antigen retrieval stained with a 1/400 dilution of polyclonal anti-DHBc antibodies (CQT-2 absorbed with NDL and NDS), and with a 1/200 dilution of secondary antibodies, as described in Section 2.7.2, showing 1.9% of DHBcAg-positive hepatocytes.

Panel (i) The liver tissue sections were photographed using 100 x magnification; Bar = 200 µm, (ii) 200 x magnification; Bar = 100 µm and (iii) 400 x magnification; Bar = 50 µm.
3.4 Production of monoclonal anti-DHBc antibodies.

3.4.1 Immunisation of mice and initial identification of hybridomas producing specific anti-DHBc antibodies.

Balb/c mice were divided into two groups of three, and were immunised with either plasmid DNA expressing DHBcAg (Group 3A) or rDHBcAg purified from *E. coli* lysates (Group 3B). In Group 3A, Balb/c mice were immunised i.m. with three doses of plasmid DNA expressing DHBcAg (100 µg of each dose) at 3 weekly intervals, followed by an i.m. boost with 50 µg of the same plasmid DNA. In Group 3B, Balb/c mice were immunised i.p. with 50 µg of purified rDHBcAg particles emulsified in FCA. Three weeks later, the mice were injected i.p. with 50 µg of antigen in FIA, followed by another two boosting doses of 50 µg without adjuvant at 3 week intervals as described in Section 2.3.3.

Blood samples were taken from each mouse before immunisation (pre-bleed) and 10 days after the third dose (test-bleed) and used to produce serum which was tested for the presence of specific antibodies by ELISA as described in Section 2.3.2.1. As shown in Figure 3.15, panel A, none of the Group 3A mice immunised with plasmid DNA expressing DHBcAg, showed any difference in the titre of anti-core antibodies in their pre-bleed and test-bleed sera (Figure 3.15; Panel A). In contrast, as shown in Figure 3.15, panel B, levels of anti-core antibodies had increased 8-10-fold in all Group 3B mice after three doses of purified rDHBcAg when compared to the pre-bleed samples. Therefore, only Group 3B mice were selected for preparation of mABs against DHBcAg. Group 3B mice were given a fourth dose of antigen (25 µg) without adjuvant via the i.p. and i.v. routes 3 days before the mice were sacrificed as described in Section 2.3.3.1.

On the day of fusion, blood samples were collected from all Group 3B mice as a positive control before the mice were sacrificed. The spleens from immunised mice were
Figure 3.15. Detection of anti-DHBc antibodies in the serum of vaccinated mice.

Balb/c mice were divided into two Groups of three, and were immunised with either plasmid DNA expressing DHBcAg (Group 3A) or rDHBcAg purified from *E. coli* lysates (Group 3B) as described in Section 2.3.3. Serum samples collected before immunisation (pre-bleed) and 10 days after the third dose (test-bleed) were screened for the presence of anti-DHBc antibodies using purified rDHBcAg coated ELISA trays as described in Section 2.3.2.1.

**Panel A.** Graph represents OD 490 nm data from Group 3A mice (Mice 3A.1, 3A.2 and 3A.3). Serum samples were titrated from 1/125 to 1/9765625.

**Panel B.** Graph represents OD 490 nm data from Group 3B mice (Mice 3B.1, 3B.2 and 3B.3). Serum samples were titrated from 1/125 to 1/9765625.
homogenized into a single cell suspension and were mixed with SP2/0 myeloma cells (Shulman et al., 1978) followed by adding feeder cell suspension as described in Section 2.3.3.1. The fusion-feeder cells suspension were grown in HA medium in either 24- or 96-well-plates to select hybridomas. 11 days after cell fusion, hybridoma clones were visible by eye and under an inverted microscope most of the wells in 24- or 96-well-plates contained at least 2-3 separate colonies with a maximum of 22 separate colonies counted in a well from a 24-well-plate. When the medium in some wells begun to turn yellow, supernatants from all wells were sampled and tested for the presence of anti-DHBc antibodies able to bind to purified rDHBcAg by ELISA as described in Section 2.3.2.1. In this ELISA, the serum samples collected just before the mice were sacrificed were used as a positive control while the pre-bleed mice serum samples collected before immunisation as well as the fresh HA medium were used as negative controls.

In total, spleens from the three Group 3B mice that were immunised with rDHBcAg were harvested to perform 3 separate fusions. The results obtained from these three fusions are presented together. In summary, a minimum of 3 separate screenings by ELISA revealed a total of 86 wells, 3 wells from 24-well-plates (42 wells plated) and 83 wells from 96-well-plates (1248 wells plated) contained antibodies able to bind rDHBcAg. The hybridomas that tested positive in ELISA were picked from their corresponding wells and were grown in larger plates. When the medium in these 86 wells turned yellow after ~2-3 days, supernatants from all wells were re-tested and 8 hybridomas having the highest antibody binding activity screened by the ELISA assay were selected. These 8 selected hybridomas, namely hybridomas 5B-11F, 1-4 5B, 2-2 10F, 2-3 8C, 2-3 5C, 2-4 8B, 1-6 1F and 2-4 11H, were subcloned by limiting dilution, twice, in round bottom 96-well-plates, resulting in 1 clone per well. This allowed the selection for the single hybridoma clone producing specific antibodies against rDHBcAg and to ensure the monoclonality. The supernatants from these 8
hybridomas were also collected to perform Western Blot screening using semi-purified rDHBcAg particles as previously described in Section 2.3.1.1.

3.4.1.1 Western Blot screening of hybridomas producing specific anti-DHBc antibodies.

The hybridomas containing specific antibodies were identified by Western Blot screening with a result of positively reacting to denatured semi-purified rDHBcAg proteins (Figure 3.16). The Western Blot screening with the eight selected hybridoma supernatants at 1/10 dilution showed that except hybridoma 2-3 5C (Figure 3.16; Lane 5), the remaining seven selected hybridomas contained specific anti-DHBc antibodies, revealing two major bands at ~33 kDa and ~64 kDa in size, corresponding to the rDHBcAg proteins and dimerisation of rDHBcAg proteins respectively. These two bands were similar to that found in the previous Western Blot assay using polyclonal rabbit anti-rDHBc antibodies (R39408, CQT-1 and CQT-2) (Figure 3.4).

Western Blot screening with supernatant from hybridoma 2-3 5C at a 1/10 dilution (Figure 3.16; Lane 5) or at 1/100 dilution (data not shown), showed high levels of background staining, indicating antibodies secreted by this hybridoma might react with E. coli proteins as the crude rDHBcAg particles used in Western blot were extracted from E. coli as described in Section 2.3.1.1. Therefore, hybridoma 2-3 5C was deleted and excluded from further analysis.

As can be seen in Figure 3.16, results from Western Blot screening showed hybridoma 5B-11F (Lane 1) secreting antibodies that reacted with semi-purified rDHBcAg particles more specific, stronger and with lower levels of non-specific background compared to the other 7 hybridomas. Therefore, after hybridoma 5B-11F was subcloned by limiting dilution twice, the single hybridoma clone of 5B-11F producing the highest amount of specific antibodies against rDHBcAg was selected by ELISA screening as described in Section 2.3.2.1. The
Figure 3.16. Western Blot screening of hybridomas producing specific anti-DHBc antibodies.

Eight hybridomas were selected based on the highest antibody binding activity screened by the ELISA assay as described in Section 2.3.2.1 and the supernatants from these eight hybridomas were collected. The presence of specific antibodies against core antigens in these eight hybridomas were confirmed using Western Blot screening, with a result of positively reacting to denatured crude purified rDHBcAg proteins. The Western Blot screening with hybridoma supernatants at a 1/10 dilution showed that except hybridoma 2-3 5C (Lane 5), the remaining seven hybridomas contained specific anti-DHBc antibodies, revealing two major bands at ~33 kDa and ~64 kDa in size, corresponding to a monomer and dimer of the rDHBcAg respectively.

Lane 1: Hybridoma 5B-11F
Lane 2: Hybridoma 1-4 5B
Lane 3: Hybridoma 2-2 10F
Lane 4: Hybridoma 2-3 8C
Lane 5: Hybridoma 2-3 5C
Lane 6: Hybridoma 2-4 8B
Lane 7: Hybridoma 1-6 1F
Lane 8: Hybridoma 2-4 11H
final clone of 5B-11F was expanded for large-scale mABs production followed by purification through a protein G affinity column at MAbSA.

Before sending to MAbSA, Western Blot screening of the supernatant collected from the final clone of 5B-11F was performed to confirm that the secreted antibodies displayed the desired antibody specificity. In this Western Blot screening, proteins extracted from LMH and tet-induced dstet5 cells as well as the semi-purified rDHBcAg particles were used (Figure 3.17). The results showed that antibodies secreted by the final clone of 5B-11F showed high specificity, reacting only with semi-purified rDHBcAg (Lane 1) and protein samples extracted from the tet-induced dstet5 cells (Lane 2), showing a specific bands at size ~33 kDa and ~64 kDa, which were specific to the DHBcAg. No band was observed in protein samples extracted from LMH cells (Lane 3). These results indicated that our final clone of 5B-11F displayed monoclonality and antibody specificity to DHBcAg.

In summary, from the 8 selected hybridomas which displayed the highest antibody binding activity screened by the ELISA, seven hybridomas secreted antibodies that bound to the rDHBcAg in Western blot and 1 hybridoma secreted antibodies that might react with E. coli protein. The hybridoma 5B-11F that secreted antibodies that bound to DHBcAg was subcloned by 2 rounds of limiting dilution and was sent to MAbSA for large-scale mABs production and purification. The remaining 6 selected un-cloned hybridomas that secreted antibodies that bound to DHBcAg were also cryopreserved.

3.4.2 Optimisation of monoclonal anti-DHBc antibodies (5B-11F).

In MAbSA, the hybridoma clones were cultured in large tissue-culture reactors and the supernatants contained large amounts of mAbs were harvested. The harvests were purified through a protein G affinity column and the final yield of mAbs was 2.1 mg/ml. The monoclonal anti-DHBc antibodies (5B-11F) obtained from MAbSA was then optimised in
Figure 3.17. Western Blot screening with the final clone of hybridoma 5B-11F to confirm that the secreted antibodies are specific to DHBcAg.

The TCA precipitated crude rDHBcAg protein, proteins extracted from LMH and tet-induced dstet5 cells (~3x10^5 cells) were used in Western Blot assay as described in Section 2.2.7. Immunoblotting with antibodies secreted by the final clone of hybridoma 5B-11F showed high specificity, reacting positively only with crude rDHBcAg proteins (Lane 1) and protein samples extracted from tet-induced dstet5 cells (Lane 2), revealing a common band at the position of ~33 kDa, corresponding to rDHBcAg. In Lane 1, bands of ~30 kDa could be the breakdown of rDHBcAg protein and the ~64 kDa band could be the dimerisation of rDHBcAg. No band was observed in protein extracted from LMH cells (Lane 3), indicating the final clone of 5B-11F displayed monoclonality and antibody specificity to DHBcAg.
Western Blot and immunostaining of liver tissue for the detection of DHBcAg as shown below.

### 3.4.2.1 Western Blot analysis using anti-DHBc mABs (5B-11F).

To confirm that the 5B-11F mAbs were specific for DHBcAg and did not cross-react with *E. coli* protein, Western Blot was performed on proteins extracted from LMH and tet-induced dstet5 cells (Figure 3.18), recombinant *E. coli* strains JM101 transformed with or without pKK233amp-DHBc (Figure 3.19), and from NDL and DHBV-infected duck liver (Figure 3.20). Four concentrations of the 5B-11F mAbs; 1/500, 1/2500, 1/12500 and 1/62500 were used. The optimum concentration of these mAbs against core antigen was 1/2500 dilution with a 1/50,000 dilution of secondary antibodies (Table 2-4).

The results showed that with the 5B-11F mAbs, specific bands at size ~33 kDa were observed in all protein samples extracted from tet-induced dstet5 cells (Figure 3.18; Lanes 2, 4, 6 and 8), JM101 with pKK233amp-DHBc (Figure 3.19; Lanes 1, 3, 5 and 7) and homogenate sample of DHBV-infected duck liver (Figure 3.20; Lane 1), corresponding to the DHBcAg. No bands were observed in protein samples extracted from LMH (Figure 3.18; Lanes 3, 5, 7 and 9), recombinant *E. coli* strains transformed with empty vector (Figure 3.19; Lanes 2, 4, 6 and 8) and an homogenate sample of NDL (Figure 3.20; Lane 2) probed with the 5B-11F mAbs at same concentration. In addition, no band was observed in homogenate sample of DHBV-infected duck liver probed with normal mouse serum (NMS) (Figure 3.20; Lane 3). These results indicated that 5B-11F mAbs did not bind to *E. coli* proteins and could recognise linear epitope on DHBcAg. The lower panel of Figures 3.18 and 3.20 showed protein loading control using Beta-actin and Figure 3.19 using Beta-lactamase.
Figure 3.18. Western blot detection of DHBcAg in tet-induced dstet5 cells using monoclonal anti-DHBc antibodies (5B-11F).

The protein from ~3x10^5 tet-induced dstet5 and LMH cells were extracted and used in Western Blot assay as described in Section 2.2.7. Four concentrations of 5B-11F mAb; 1/500, 1/2500, 1/12500 and 1/62500 were used. The optimum concentration of 5B-11F mAb against core antigen was a 1/2500 dilution with a 1/50,000 dilution of secondary antibodies (Table 2-4).

**Upper panel:** Immunoblotting with 5B-11F mAb revealed a sharp band at the position of ~33 kDa with protein extracted from tet-induced dstet5 cells, corresponding to the DHBcAg (Lanes 2, 4, 6 and 8). This band was not observed in protein extracted from LMH cells probed with same antiserum at same concentration (Lanes 1, 3, 5 and 7).

**Lower panel:** The intensity of β-actin specific bands was similar in all tested samples suggesting that similar amounts of protein were loaded in each lane of the gel.

Lane 1: LMH  
Lane 2: dstet5  
Lane 3: LMH  
Lane 4: dstet5  
Lane 5: LMH  
Lane 6: dstet5  
Lane 7: LMH  
Lane 8: dstet5

- 5B-11F mAb at a 1/62500 dilution
- 5B-11F mAb at a 1/12500 dilution
- 5B-11F mAb at a 1/2500 dilution
- 5B-11F mAb at a 1/500 dilution
Figure 3.19. Western blot analysis of DHBcAg expression in *E. coli* strain JM101 transformed with pKK223amp-DHBc using monoclonal anti-DHBc antibodies (5B-11F).

To confirm that the mAb 5B-11F are specific for DHBcAg and do not cross-react with *E. coli* protein, proteins extracted from JM101 transformed with pKK233amp-DHBc (a recombinant *E. coli* strain that was used to produce rDHBcAg) and JM101 transformed with pKK223amp (a control *E. coli* strain) were used in Western Blot assay as described in Section 2.2.7. Four concentrations of 5B-11F mAb; 1/500, 1/2500, 1/12500 and 1/62500 were used. The optimum concentration of 5B-11F mAb against core antigen was 1/2500 dilution with a 1/50,000 dilution of secondary antibodies (Table 2-4).

**Upper panel:** Immunoblotting with mAb 5B-11F revealed a sharp band at the position of ~33 kDa with protein extracted from JM101 transformed with pKK233amp-DHBc, corresponding to the DHBcAg (Lanes 1, 3, 5 and 7). This band was not observed in protein extracted from the control *E. coli* strain probed with the same mAb at same concentration (Lanes 2, 4, 6 and 8).

**Lower panel:** The intensity of beta-lactamase bands was similar in all tested samples suggesting that similar amounts of protein were loaded in each lane of the gel.
Lane 1: JM101 + pKK233amp-DHBc 5B-11F mAb at a 1/500 dilution
Lane 2: JM101 + pKK233amp 5B-11F mAb at a 1/2500 dilution
Lane 3: JM101 + pKK233amp-DHBc 5B-11F mAb at a 1/12500 dilution
Lane 4: JM101 + pKK233amp 5B-11F mAb at a 1/62500 dilution
Lane 5: JM101 + pKK233amp-DHBc 5B-11F mAb at a 1/12500 dilution
Lane 7: JM101 + pKK233amp-DHBc 5B-11F mAb at a 1/62500 dilution

Lane 8: JM101 + pKK233amp 5B-11F mAb at a 1/62500 dilution
Figure 3.20. Western blot analysis of DHBcAg expression in NDL and DHBV-infected duck liver using monoclonal anti-DHBc antibodies (5B-11F).

50 mg of liver tissue from a DHBV-infected duck and a non DHBV-infected duck (NDL) was homogenised separately in lysis buffer (Table 2-3). The protein in the homogenate supernatant was then collected, denatured and mixed with loading buffer before being analysed by Western Blot as described in Section 2.2.7.

**Upper panel:** Immunoblotting with mAb 5B-11F at a 1/1000 dilution revealed a sharp band at the position of ~33 kDa with protein extracted from DHBV-infected duck liver, corresponding to the liver derived DHBcAg (Lane 1). This band was not observed in the homogenate samples from NDL (Lane 2) that probed with same mAb at same concentration or in homogenate sample probed with a 1/1000 dilution of NMS (Lane 3).

**Lower panel:** The intensity of β-actin specific bands was similar in all tested samples suggesting that similar amounts of protein were loaded in each lane of the gel.

Lane 1: DHBV-infected duck liver probed with 5B-11F mAb at a 1/1000 dilution
Lane 2: NDL probed with 5B-11F mAb at a 1/1000 dilution
Lane 3: DHBV-infected duck liver probed with NMS at a 1/1000 dilution
3.4.2.2 Detection of DHBcAg-positive hepatocytes in liver tissue sections using the 5B-11F mAbs

To detect DHBcAg-positive hepatocytes, immuno-staining with the 5B-11F mAbs was performed on 6-week-old duck liver tissue fixed in EAA (slide # 4871) and formalin (slide # 4872). The 5B-11F mAbs were used at five different concentrations; 1/100, 1/200, 1/500, 1/1000 or 1/1500 and the concentration of the secondary antibodies sheep-anti-mouse was 1/250.

As can be seen in Figure 3.21, no DHBcAg-positive hepatocytes were detected in EAA-fixed tissue (slide # 4871) with or without antigen retrieval (Panel A and B) and in the control EAA-fixed tissue (slide # E64388) (Panel C). The failure to detect any DHBcAg-positive hepatocytes in slide # 4871 was probably due to the inefficient of EAA fixative to retain or preserve the antigens in the cell for antibody binding.

In contrast, DHBcAg-positive hepatocytes were detected in formalin-fixed tissues (slide # 4872) with antigen retrieval but not in the non DHBV-infected formalin-fixed tissue (slide # 6122) (Figure 3.22). The optimum concentration of 5B-11F mAbs in formalin-fixed tissues with antigen retrieval was 1/100 combined with a 1/250 dilution of the sheep anti-mouse secondary antibodies.

3.5 Expression of the DHBV DNA vaccines in vitro.

In this project, DHBV DNA vaccines expressing DHBV pre-S/S, S and C were cloned into the DNA vaccine vector pcDNA3 by Dr Feng Feng (Feng et al., 2010), to replace the similar pcDNA1.1-based DNA vaccines used in previous studies performed in our laboratory. The ability of these constructs to express DHBV pre-S/S and C proteins were examined by indirect IMF assay following transfection of 293T cells as described in Section 2.2.3 and by Western Blot following electroporation of PDEF as described in Section 2.2.5. Currently, the
Figure 3.21. Immuno-staining of EAA-fixed liver sections for the detection of DHBcAg-positive hepatocytes.

**Panel (A)** EAA-fixed liver tissue sections from a 6-week-old DHBV-infected duck (slide # 4871) with antigen retrieval stained with monoclonal anti-DHBc antibodies (5B-11F), and (B) without antigen retrieval, showing no positive hepatocytes at a 1/100 dilution.

**Panel (C)** EAA-fixed liver tissue sections from a 6-week-old non DHBV-infected duck (slide # E64388) stained with monoclonal anti-DHBc antibodies (5B-11F) showing no positive hepatocytes at a 1/100 dilution.

**Panel (i)** The liver tissue sections were photographed using 200 x magnification; Bar = 100 µm and (ii) 400 x magnification; Bar = 50 µm.
Figure 3.22. Immuno-staining of formalin-fixed with antigen retrieval liver section for the detection of DHBcAg-positive hepatocytes.

**Panel (A)** Formalin-fixed liver tissue sections from a 6-week-old DHBV-infected duck (slide # 4872) with antigen retrieval stained with monoclonal anti-DHBc antibodies (5B-11F) at a 1/100 dilution, showing 0.44% of DHBcAg-positive hepatocytes.

**Panel (B)** Formalin-fixed liver tissue sections from a 6-week-old non DHBV-infected duck (slide # 6122) with antigen retrieval stained with monoclonal anti-DHBc antibodies (5B-11F) showing no positive hepatocytes at a 1/100 dilution.

**Panel (i)** The liver tissue sections were photographed using 200 x magnification; Bar = 100 µm and (ii) 400 x magnification; Bar = 50 µm.
available anti-S monoclonal antibodies (7C.12) (Pugh et al., 1995) only detect DHBV-16 (USA strain of DHBV) and polyclonal rabbit anti-DHBs antibodies (Qiao et al., 1990) is the only reagent available in our laboratory to detect DHBV S. However, the polyclonal rabbit anti-DHBs antibodies to detect S protein were not optimal for Western Blot and IMF assays, constructs expressing DHBV S were not studied in here.

In IMF assays, similar levels of expression of DHBV pre-S/S and C antigens were observed in transfected 293T cells at three days post-transfection using anti-DHBV pre-S/S mABs (Pugh et al., 1995) and polyclonal anti-DHBc antibodies (CQT-2) respectively (Table 2–2). The transfection efficiency of pre-S/S cloned in pcDNA3 and pcDNA1.1 constructs was also similar, resulting in ~6.9% and ~6.3% DHBsAg-positive cells respectively (Figure 3.23; Panel A and B). For core protein, the transfection efficiency of pTC-Dcore (~11.8%) was slightly higher than pcDNA3-C (~8.9%) in 293T cells (Figure 3.24; Panel A and B). No specific IMF was seen in 293T cells transfected with the parental plasmid (Figure 3.23 and 3.24; Panel C).

Following electroporation of PDEF, Western Blot assay using the appropriate antibodies (Table 2-4) was performed. At three days post-transfection, pre-S/S protein at size ~36 kDa and ~28 kDa and C protein at size ~33 kDa were detected in a lysate of PDEF transfected with constructs expressing DHBV pre-S/S and C respectively (Figure 3.25). The protein expression of pre-S/S in pcDNA3 construct was slightly stronger than pcDNA1.1 construct (Figure 3.25, Panel A: Lanes 1 and 2). The DHBcAg expression in PDEF transfected with pTC-Dcore was stronger than in PDEF transfected with pcDNA3-C (Figure 3.25, Panel B: Lanes 2 and 4). No specific DHBcAg band was seen in PDEF transfected with the parental plasmid (Figure 3.25: Panel A: Lanes 3 and 4; Panel B: Lanes 1 and 3). The intensity of β-actin specific bands (~42 kDa) appeared at similar levels in all tested samples suggesting that
Figure 3.23. IMF analysis of DHBV antigen expression in 293T cells transfected with DHBV DNA vaccines expressing pre-S/S antigens.

293T cells were transfected with DHBV DNA vaccine expressing pre-S/S antigens as described in Section 2.2.3. Three days post-transfection, specific IMF in the transfected 293T cells was observed when monoclonal anti-pre-S/S antibodies (1H.1) (Pugh et al., 1995) were used as primary antibodies as described in Section 2.2.6. No IMF was seen in 293T cells transfected with the parental plasmids.

Panel (i) 293T cells transfected with DHBV DNA vaccine expressing pre-S/S antigens counter-stained with DAPI to detect cell nuclei, (ii) the same transfected 293T cells stained with FITC-labelled antibodies to detect the cytoplasmic DHBsAg expression and (iii) A merge image of the same transfected 293T cells stained with DAPI and FITC using imaging software. All photos were taken at 200 x magnification.

Panel A. 293T cells transfected with pcDNA1.1-pre-S/S, showing 6.3% DHBsAg-positive cells

Panel B. 293T cells transfected with pcDNA3-pre-S/S, showing 6.9% DHBsAg-positive cells

Panel C. 293T cells transfected with pcDNA1.1.
Figure 3.24. IMF analysis of DHBV antigen expression in 293T cells transfected with DHBV DNA vaccines expressing core antigens.

293T cells were transfected with DHBV DNA vaccines expressing core antigens, either with pTC-Dcore (Von Weizsacker et al., 1995) or pcDNA3-C (Feng et al., 2010), as described in Section 2.2.5. Three days post-transfection, specific IMF in the transfected 293T cells was observed when polyclonal anti-DHBc antibodies (CQT-2) were used as primary antibodies as described in Section 2.2.6. No IMF was seen in 293T cells transfected with the parental plasmids.

Panels (i) 293T cells transfected with DHBV DNA vaccine expressing core antigens counter-stained with DAPI to detect cell nuclei, (ii) the same transfected 293T cells stained with FITC-labelled antibodies to detect cytoplasmic DHBcAg expression and (iii) a merged image of the same transfected 293T cells stained with DAPI and FITC using imaging software. All photos were taken at 200 x magnification.

Panel A. 293T cells transfected with pTC-Dcore, showing 11.8% DHBcAg-positive
Panel B. 293T cells transfected with pcDNA3-C, showing 8.9% DHBcAg-positive
Panel C. 293T cells transfected with pcDNA3
Figure 3.25. Expression of pre-S/S and DHBcAg proteins in PDEF by Western Blot.

Electroporation was used for transfection of PDEF as described in Section 2.2.5. After 48 hr post-transfection, the proteins (~5x10^6 cells) were extracted and used in Western Blot assay as described in Section 2.2.7.

**Panel (A)** Western Blot analysis of PDEF cell lysates for the expression of pre-S/S antigens using monoclonal anti-pre-S/S antibodies (1H.1) (Pugh et al., 1995) (Table 2-4). Two sharp bands at the position of ~36 kDa and ~28 kDa were observed with protein extracted from PDEF cell lysates (Lanes 1 and 2). The sharp band at the position of ~36 kDa, corresponding to the full-length pre-S/S proteins while another band at the position of ~28 kDa, corresponding to a product from an internal initiation of translation or post-translational degradation, which has previously been described in DHBV-infected liver (Triyatni et al., 2001). These bands were not observed in protein extracted from PDEF transfected with parental plasmids that were probed with the same antibodies at the same concentration (Lanes 3 and 4).

**Panel (B)** Western Blot analysis of PDEF cell lysates for the expression of core antigens using polyclonal anti-DHBc antibodies as primary antibodies (Table 2-4). A sharp band at the position of ~33 kDa with protein extracted from PDEF cell lysates, corresponding to the core protein (Lanes 2 and 4). This band was not observed in protein extracted from PDEF transfected with parental plasmids that were probed with the same antibodies at the same concentration (Lanes 1 and 3).

**Panel A and B (Lower panel):** The intensity of β-actin specific bands (~42 kDa) was similar in all tested samples suggesting that similar amounts of protein were loaded in each lane of the gel.
A) pcDNA3-pre-S/S  pcDNA1.1-pre-S/S  pcDNA3  pcDNA1.1

37 kDa  ➔  Pre-S/S
26 kDa  ➔
42 kDa  ➔  β-actin

B) pcDNA3  pcDNA3-C  pcDNA1.1  pTC-Deore

37 kDa  ➔  DHBcAg
26 kDa  ➔
42 kDa  ➔  β-actin
the same amount of protein was loaded in each well of the gel (Figure 3.25: Panel A and B (Bottom)).

### 3.6 Discussion

One of the aims of this project was to generate specific antibodies that can facilitate the detection of DHBcAg in the liver of DHBV-infected ducks. The attempts at producing polyclonal and mABs against DHBcAg were overall successful. Testing of sera by ELISA revealed strong specific antibody responses in rabbits following 3 immunisations with rDHBcAg proteins. Polyclonal antisera against core antigen (CQT-1 and CQT-2) were collected from rabbits after the fourth immunisation. For mABs production, the ELISA screening of hybridoma supernatants revealed a total of 86 wells positive for antibodies that reacted with DHBcAg. This indicated immunisation of purified rDHBcAg proteins in mice was successful in generating specific antibody producing B lymphocytes to produce high numbers of hybridomas after cell fusions. The hybridoma 5B-11F was selected to be subcloned by limiting dilution and the final clone of 5B-11F was sent to MAbSA for mass production.

To prepare mAbs against DHBcAg, only mice that were immunised with rDHBcAg purified from *E. coli* lysates (Group 3B) but not with plasmid DNA expressing DHBcAg (Group 3A) were selected. The ELISA results showed that the antibody titres of the immune sera from the Group 3B mice were ~7-8-fold higher than the Group 3A mice, indicating that the DNA vaccine was not as efficient as a high concentration of recombinant protein in inducing specific antibody responses in mice (Figure 3.15). This could be due to the low gene expression of plasmid DNA delivered to muscle tissues. DNA immunisation of mice by alternative route e.g., intrasplenic immunisation, could be more effective than i.m. immunisation (Velikovsky *et al.*, 2000). However, such alternative routes could be difficult to perform in mice. In addition, the purified rDHBcAg proteins could be given following DNA
immunisation to boost specific antibody response in mice before the fusion step. However, this was not performed in Group 3A mice due to the shortage of purified rDHBcAg proteins.

The immuno-staining with the polyclonal antisera (CQT-2) or the 5B-11F mAbs showed similar distribution patterns of DHBcAg in duck liver tissue sections and the DHBcAg was found in the cytoplasm of hepatocytes. In addition, the immuno-staining of core antigens showed higher staining intensity and had more detectable DHBcAg-positive hepatocytes when formalin-fixed tissue with antigen retrieval was used compared to EAA-fixed tissue with or without antigen retrieval. This is not a surprise as formalin-based fixative is known to preserve protein targets and tissue morphology better than EAA-based fixative. In addition, the EAA-based fixative is primarily used to fix membrane surface antigens as it does not penetrate as well as formalin-based fixative (rndsystems, Technical Information, 2013). This might explain the overall superiority of immuno-staining to detect DHBcAg in formalin-fixed liver tissue after the antigen retrieval process, as compared to EAA-fixed liver tissue. Nonetheless, the tissue handling steps (such as the fixation time, fixative composition and storage after fixation) and antigen retrieval steps can also affect the antibody binding in immuno-staining assay.

However, polyclonal anti-DHBc antibodies (CQT-2) were more sensitive in immuno-staining for detection of DHBcAg than the 5B-11F mAbs prepared from the same stock of rDHBcAg. In formalin-fixed tissues with antigen retrieval, staining with the 5B-11F mAbs had less detectable DHBcAg-positive hepatocytes and weaker intensity (Figure 3.22) in comparison to staining with polyclonal antisera CQT-2 (Figure 3.13). This is not surprising as the polyclonal antiserum consists of a mixture of antibodies that recognize different epitopes and will thus bind to a higher number of targets. This strong interaction between the polyclonal antibodies and the corresponding antigen, results in more sensitive and higher intensity of DHBcAg-positive staining cells.
Other than the detection of the core antigen as fixed antigens in EAA- and formalin-fixed hepatocytes in liver sections and as a native antigen in ELISA, the polyclonal antisera (CQT-1 and CQT-2) and 5B-11F mAbs also reacted positively and specifically in Western Blot assay. This suggests that the polyclonal antisera (CQT-1 and CQT-2) and the 5B-11F mAbs can target a linear epitope on denatured or partially denatured core antigens. In addition, specific IMF also detected in tet-induced dstet5 cells probed with polyclonal antisera (CQT-1 and CQT-2), again further confirmed that these polyclonal antisera could bind to native form of core antigens. This was not a surprise for polyclonal antisera because different components of the antisera might bind to different formats and different epitopes on the same antigens (Burry, 2010). In contrast, although it is unusual for mAbs to bind antigens in a range of formats, mAbs that can bind to both denatured and native antigens have been previously reported against adenovirus DNA-binding protein (Reich et al., 1983); against luciferase, (Xu et al., 2004); and against vascular endothelial growth factor (Hu et al., 2009).

The immuno-staining of two different antigens of HBV: surface and core antigens in hepatocytes is equally important in confirming the presence of HBV infection and indicating active virus replication in liver of HBV-infected patients (Gowans and Burrell, 1985), as is seen in liver of DHBV-infected ducks (Jilbert et al., 1992). In our duck model, the immuno-staining to detect DHBsAg- and DHBcAg-positive hepatocytes was optimised in EAA- and formalin-fixed liver tissue sections respectively. EAA is a good fixative which does not alter the structure of lipid membrane and tissue morphology (Baker, 1958). DHBsAg is a transmembrane protein, hence explains the detection of DHBsAg was optimised in EAA-fixed tissue section, showing more sensitive and stronger staining intensity compared to DHBcAg using the same fixative.

For comparison, immuno-staining for the detection of DHBsAg and DHBcAg was performed on liver samples collected from a same duck at the same time-point. The results showed the
detection of DHBsAg and DHBcAg has similar sensitivity, which detected 2.2% and 1.9% of positive staining hepatocytes respectively, allowing cross-checking with each other (Figure 3.14). In general, DHBsAg and DHBcAg showed similar distribution patterns within the liver lobules of DHBV-infected ducks and were detected in the cytoplasm of hepatocytes only. Interestingly, in chronically HBV-infected humans, HBcAg was found in both nuclei and cytoplasm of hepatocytes, indicating active virus replication (Kakumu et al., 1989). It has been known that phosphorylation of core protein induces exposure of the nuclear localization signal (NLS). This results in the binding of core protein to the nuclear pore complexes (NPC) and subsequently the transportation of the capsid containing the viral genome to the nucleus, an important step for HBV replication (Kann et al., 1999). However, it has been shown that the substitution of the phosphorylation site in DHBV core sequence had no effect on the distribution of core protein, which was predominantly cytoplasmic (Yu and Summers 1994). This indicated DHBV might have different mechanism in regulating the distribution/localisation of core antigen and possibly explained the absence of DHBcAg staining in nuclei of hepatocytes.

The pcDNA3 vector is a direct replacement of the low yield pcDNA1.1 vector. To standardise and increase the yield of DNA vaccines, DHBV DNA vaccines expressing DHBV- pre-S/S, -S and -C were cloned in pcDNA3 constructs by Dr Feng Feng (Feng et al., 2010), to replace the previous DHBV DNA vaccines in our laboratory. The ability of these pcDNA3 constructs to express the cloned DHBV antigens were confirmed by indirect IMF assay and Western Blot using appropriate antibodies. The IMF assay showed that all constructs had similar expression intensity and the higher transfection efficiency in constructs expressing C in comparison to constructs expressing pre-S/S was probably due to the higher background in the latter (Figures 3.23 and 3.24). The western blotting of PDEF lysates demonstrated that protein expression levels of pcDNA3 constructs was slightly better for pre-S/S but not for C antigens (Figures 3.25). Nonetheless, the newly made pcDNA3-
based DHBV DNA vaccines showed significant transfection efficiency and protein expression levels. Therefore, pcDNA3-based DHBV DNA vaccines were used in studies in Chapter 4, 5 and 6.
Chapter 4: Assessment of a duck CD40 ligand (DuCD40L) DNA construct as a genetic adjuvant for DHBV DNA vaccines

4.1 Introduction and aims

The current protective HBV vaccine, which contains the small HBV envelope proteins induces anti-HBs antibodies in 80-95% of vaccinated recipients and provides protection from HBV infection (Mast et al., 2005). However, the vaccine provides no therapeutic benefit in patients with CHB. Therefore, there is an urgent need to develop new therapeutic vaccines for chronic HBV that might include adjuvants to enhance their efficacy.

Inoculation of DNA vaccines that encode specific proteins of interest has been proven to induce both humoral and CMI, including activation of antigen specific CTL particularly when the i.m. route of delivery is used (Donnelly et al., 1997). Since chronic HBV infection has been characterised as being associated with weak or unresponsive CD4+ and CD8+ T cell responses against HBV antigens (Wieland et al., 2005), DNA-based vaccines that can induce both humoral and CMI have been considered as potential components of therapeutic vaccines for CHB. After inoculation of a DNA vaccine by the i.m. route, DNA vaccines are preferentially taken up by APCs, rather than myocytes or keratinocytes (Torres et al., 1997). This could be due to the direct transfection of APCs or cross-priming of APCs when DNA vaccine-transfected somatic cells are engulfed by APCs (Ulmer et al., 1996; Rajcani et al., 2005; Belakova et al., 2007). After DNA vaccination, the APCs process and present these DNA-vaccine expressed antigens in the context of both MHC-I and MHC-II molecules at the cell surface to initiate both T and B cell responses (Gurunathan et al., 2000; Liu, 2003; Belakova et al., 2007).

Initial studies performed in hepadnavirus animal models, including the chimpanzee, transgenic mouse, woodchuck and duck models have shown that DNA vaccines expressing hepadnavirus
antigens are effective in activating both humoral and CMI against cloned virus antigens (Triyatni et al., 1998; Lu et al., 1999; Rollier et al., 1999; Xing et al., 2005; Miller et al., 2006; Shata et al., 2006; Saade et al., 2008; Thermet et al., 2008; Yao et al., 2010; Saade et al., 2013). However, the immunogenicity of therapeutic DNA vaccines in non-human primates and CHB patients remained relatively low compared to those in small animal models, especially in regard to Th1 immune responses that are likely to be required to mount effective immune responses against HBV infection (Shata et al., 2006; Mancini-Bourgine et al., 2004, 2006). Therefore, improvement of the immunogenicity and protective efficacy of DNA vaccines is the major concern for the development of immunotherapy for CHB infection. Our recent immunotherapy approaches in the DHBV model (Miller et al., 2006, 2008; Feng et al., 2010) have demonstrated a promising effect in targeting DHBV-infected hepatocytes but could not fully clear widespread DHBV infection suggesting more effective vaccination is required for the resolution of persistent DHBV infection. In the study described in this chapter, DHBV DNA vaccines were co-administered with a CD40L expression construct to strengthen the immune effect of DNA vaccine in our immunotherapy approaches.

CD40L, also known as CD154, is a type II integral membrane glycoprotein and a member of TNF-α family of cell surface interaction molecules (Daoussis et al., 2004). CD40L is mainly expressed on the surface of activated CD4+ T cells, but also on other cell types, such as B lymphocytes, NK cells and endothelial cells. On the other hand, its receptor, CD40, is constitutively expressed mainly on APC, including B lymphocytes, macrophages and DC (Blossom et al., 1999; Higuchi et al., 2002; Jyothi et al., 2000; Mach et al., 1997).

The CD40L on CD4+ T lymphocytes has been shown to license DC via CD40 to prime CTL responses (Bennett et al., 1998; Smith et al., 2004). The CD40-signalling also results in improved interaction between B and CD4+ T lymphocytes. It promotes B lymphocyte differentiation and proliferation and immunoglobulin isotype switching (Garside et al., 1998;
Sun et al., 2003; Smith et al., 2004; Daoussis et al., 2004; Fraser et al., 2007). In general, the expression of CD40L is transient which means CD40-signalling only has a limited time to deliver helper signals to APC (Fuleihan et al., 1994; Fraser et al., 2007). Thus, CD40L plays a critical role as a co-stimulatory molecule in the CD40-signalling pathway that involves activation of APC and of both humoral and CMI responses.

Currently, a number of studies involve in the administration of CD40L as an immunological adjuvant in anti-tumor and antiviral therapies. These include: (1) CD40L expressed by plasmid DNA constructs (Stone et al., 2006; Yao et al., 2010), (2) CD40L expressing APCs as immunogens (von Bergwelt-Baildon et al., 2002), (3) CD40L as a fusion protein (Huang et al., 2004; Gomez et al., 2009) (4) CD40L expressed by poxvirus vectors (Feder-Mengus et al., 2005; Liu et al., 2008) or (5) CD40L expressed by adenoviruses (Peter et al., 2002). A recent study performed by Yao et al., 2010 showed that DNA constructs expressing CD40L significantly improved the humoral immune response to the vector-encoded avian influenza hemagglutinin (HA) protein and provided partial protection in challenge experiments in ducks. Recently, the duck homologue of CD40L (DuCD40L) was cloned and was shown to exhibit immunological adjuvant characteristics similar to human CD40L (Fischer et al., 2006). Further studies by the same group demonstrated that ducks immunised with DNA vaccines expressing a chimera of DuCD40L fused to a truncated form of DHBV core antigen (tcore-DuCD154) exhibited accelerated and stronger core antigen-specific humoral immune responses compared to the control group of ducks immunised with plasmid DNA expressing DHBV core antigen alone (Gares et al., 2006). However, in this study the vaccinated ducks were not challenged with DHBV and thus the protective efficacy of DNA vaccines expressing DuCD40L could not be determined.

In this Chapter, the protective efficacy of DNA vaccines expressing DHBV antigens, co-administered with DuCD40L expression construct was tested by vaccination of ducks and then
challenge with $4.5 \times 10^{10}$ DHBV genomes (Figure 4.1). This virus dose was based on previous studies, where inoculation of two 42-day-old ducks with this virus dose had lead to DHBV infection in ~30% of hepatocytes in the liver by day 4 p.c. Following challenge one duck subsequently developed acute DHBV infection, while the other duck developed persistent DHBV infection (Reaiche, Ph.D. thesis, 2008).

4.2 Experimental design

4.2.1 Experimental design of *in vivo* studies

In this study, 4 DNA plasmid constructs were used: pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C and pcDNA3-DuCD40L (or pCDuCD40L). The pcDNA3-pre-S/S, pcDNA3-S and pcDNA3-C plasmid constructs were previously described in Feng et al., 2010. The ability of these constructs to express DHBV pre-S/S, S and C proteins was confirmed by electroporation of PDEF followed by western blot as described in Section 2.2.7 using the appropriate antibodies as listed in Table 2-4.

All ducks were bled weekly and serum samples were analysed for levels of DHBsAg by rapid qualitative ELISA as described in Section 2.6.1; (ii) the levels of anti-DHBs antibodies in the serum were measured by quantitative ELISA as described in Section 2.6.2; (iii) the levels of anti-DHBc antibodies in the serum were measured by quantitative ELISA as described in Section 2.6.3; and (iv) serum DHBV DNA was extracted using the ChargeSwitch gDNA 1 mL serum kit as described in Section 2.8.1 and served as templates for serum DHBV DNA qPCR assay using primer set 423 – 567c (Table 2-1) as described in Sections 2.8.3 and 2.8.4.
Figure 4.1. The schedule of DNA vaccination, virus challenge, and liver biopsies and autopsies. In Experiments I and II, nineteen and ten, 14-day-old ducks were vaccinated i.m., at 14 and 28 days of age. All ducks were vaccinated with 500 µg of each DHBV DNA vaccine construct: Group 4A, DHBV DNA vaccines (pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C) co-administrated with pcDuCD40L; Group 4B, DHBV DNA vaccines alone; Group 4C and 4E, pcDuCD40L alone; Groups 4D and 4F, pcDNA3 vector. All ducks (except Group 4D ducks) were challenged at 42 days of age with $4.5 \times 10^{10}$ DHBV genomes. Liver biopsies were performed at day 4 and 14 p.c. with autopsies at day 31 p.c. The experimental groups in Experiment I and II are summarised in Table 4-1.
Table 4-1. The summary of experimental groups in Experiments I and II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>DHBV vaccine$^a$</th>
<th>pcDuCD40L</th>
<th>pcDNA3 vector</th>
<th>DHBV challenge</th>
<th>Number of ducks</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4A</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>4B</td>
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<td></td>
<td>4C</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>6</td>
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<tr>
<td></td>
<td>4D$^c$</td>
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<td>-</td>
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<tr>
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<td>4F</td>
<td>-</td>
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<td>+</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$: Ducks were vaccinated with DHBV DNA vaccines that contained a mixture of pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C with 500 µg of each construct as described in Section 2.5.2.1.

$^b$: All ducks (except Group 4D ducks) in Experiment I and II were challenged at 42 days of age with $4.5 \times 10^{10}$ DHBV genomes. This virus dose was chosen based on previous studies, where inoculation of two 42-day-old ducks with this virus dose had lead to DHBV infection in ~ 30% of hepatocytes in the liver by day 4 p.c., with one duck subsequently developed acute DHBV infection, while the other duck developed persistent DHBV infection (Reaiche Ph.D. thesis, 2008).

$^c$: Three Group 4D ducks were vaccinated i.m. with pcDNA3 vector, twice, at 14 and 28 days of age. Ducks in this group were not challenged with DHBV.
Liver tissues samples obtained by biopsy (day 4 and 14 p.c.) and autopsy (day 31 p.c.) of each duck were used for: (i) immuno-staining of DHBsAg in sections of EAA-fixed duck liver using monoclonal anti-DHBV pre-S/S antibodies, 1H.1 (Pugh et al., 1995) to determine the percentage of virus antigen positive hepatocytes as described in Section 2.7.1. and (ii) qPCR analysis of total DHBV DNA after total DNA extraction using DNeasy® Blood and tissue kit as described in Section 2.8.2 and qPCR using primer set 423 – 567c (Table 2-6) as described in Sections 2.8.3 and 2.8.5.

**Results**

**4.3 In vitro Results**

All techniques described below in Sections 4.3.1 – 4.3.3 were performed by Dr Feng Feng in the Hepatitis Virus Research Laboratory and are presented here for completeness.

**4.3.1 Isolation and Sequence Analysis of DuCD40L**

The DuCD40L cDNA was synthesised from total RNA extracted from duck PBMC and splenocytes as described in Section 2.4.1.3. By using specific primers for detection of DuCD40L mRNA (Table 2-6), the DuCD40L cDNA generated by RT PCR was the expected size of ~ 820 bp (Figure 4.2). These specific primers (Table 2-6) were designed based on the reported duck CD40L sequence (Genbank Accession DQ267671). The upstream primer of DuCD40L contained a BamHI RE digestion site and a Kozak sequence (Table 2-6). The downstream primer of DuCD40L contained an XhoI RE digestion site and a Flag epitope sequence (Table 2-6). After the DNA fragment was digested with BamHI and XhoI and was subsequently subcloned into BamHI and XhoI sites of pcDNA3 downstream of CMV promoter as described in Section 2.4.1.5., the resultant construct was designated as pcDuCD40L. The cloning of the DuCD40L cDNA into the vector was confirmed by BamHI and XhoI double digestion (Figure not shown). The pcDuCD40L cDNA was transformed into
Figure 4.2. RT-PCR amplification of the DuCD40L cDNA sequence using mRNA extracted from duck PBMC and splenocytes

The DuCD40L gene was amplified with specific primers listed in Table 2-6. The PCR products were analyzed on a 1.2% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA). Lane 1 is the GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific). Lane 2 shows that the cDNA product generated by RT PCR using primers for detection of DuCD40L mRNA was of the expected size, ~ 820 bp (black arrow).
**E. coli** DH5α as described in Section 2.4.1.6 and was purified using the Jetstar Maxi kit as described in Section 2.4.1.7. Purified pcDuCD40L DNA was used in both *in vitro* and *in vivo* studies in this Chapter.

Further confirmation of the DuCD40L clone was undertaken by sequencing and resultant sequences were compared to CD40L cDNA sequences of the duck (GenBank Accession DQ267671), chicken (GenBank Accession AJ243435) and human (GenBank Accession P29965) homologues using BioEdit software (Figure 4.3). The reported DuCD40L sequence was previously published by a Canadian group (Fischer et al., 2007). The alignment of deduced amino acid sequences revealed that the Australian Pekin duck DuCD40L clone (Pekin (Aus)) (GenBank Accession EU918428) shared 99% of sequence homology to the reported Canadian Pekin duck CD40L (Pekin (Ca)) with only two AA differences occurring at positions 4 and 270, with alanine (A) and methionine (M) found in DQ267671 being replaced in our clone by valine (V) and isoleucine (I) respectively. The A, M, V and I all have non-polar side chains, indicating that these AA share similar properties. This suggests our cloned Pekin (Aus) DuCD40L retains the structural features of the reported Pekin (Ca) DuCD40L (Fischer et al., 2007), which includes the entire N-terminal intracellular domain, transmembrane region and a C-terminal secretory leader sequence. The results also showed that the Pekin (Aus) DuCD40L clone had approximately 83% and 41% AA sequence homology to chicken and human CD40L sequences respectively.

### 4.3.2 Confirmation of DuCD40L protein expression by Western Blot detection in LMH cells transfected with pcDuCD40L

To confirm expression of DuCD40L in LMH cells transfected with pcDuCD40L as described in Section 2.2.5., at 48 hr post-transfection, cellular proteins were harvested by lysis of the LMH cells and Western blots was probed with anti-Flag monoclonal antibodies (Table 2-4) as described in Section 2.2.7. The expression of Flag-tagged DuCD40L protein was detected by
Figure 4.3. Alignment of the deduced AA sequences of the DuCD40L protein with the reported AA sequences of the Pekin duck, chicken and human CD40L.

The Pekin (Aus) is the DuCD40L AA sequences determined in this study while Pekin (Ca) is the duck CD40L sequence reported by a Canadian group (Fischer et al., 2007). Alignment of 3 deduced AA sequences revealed that the Pekin (Aus) DuCD40L (GenBank Accession EU918428) shared 99% of sequence homology to the reported Pekin (Ca) DuCD40L (GenBank Accession DQ267671) with only two AA differences at positions 4 and 270, with A and M found in DQ267671 being replaced in clone by V and I respectively. The results also showed that our DuCD40L clone had approximately 83% and 41% homology to the chicken (GenBank Accession AJ243435) and human CD40L (GenBank Accession P29965) sequences respectively.
Western Blot as a band of ~ 33 kDa (lane 2 of Figure 4.4). The DuCD40L expression was not detected in LMH cells transfected with the pcDNA3 control plasmid (lane 1 of Figure 4.4) or the Flag vector control plasmid (lane 3 of Figure 4.4) indicating the construct pcDuCD40L expresses a Flag-tagged protein. To determine if the same amount of total protein was loaded in each well, anti-human beta actin polyclonal antibodies (See Table 2-4) were used as a loading control in Western Blot. A ~ 42 kDa band was detected at a similar level in all samples, suggesting that protein samples were loaded at a similar level (bottom panel of Figure 4.4) as previously described (Feng et al., 2010).

4.3.3 DuCD40L induced Nitric Oxide (NO) production in HD11 cells which was accompanied by inducible nitric oxide synthase (iNOS) gene up-regulation

It was previously shown that interaction between CD40 on macrophages and CD40L on activated T lymphocytes results in the secretion of cytokines such as TNF-α, IL-6, IL-10 and iNOS gene expression in murine and human macrophages (Kiener et al., 1995; Tian et al., 1995; Sommer et al., 2009). An increase of iNOS protein expression in human macrophage cells results in the production of NO, contributing to the killing of various microbes (Munoz-Fernandez et al., 1992; Vouldoukis et al., 1995). In other species such as rabbits and mice (Tian et al., 1995; Bingaman et al., 2000; Molero et al., 2005) CD40L has also been shown to induce NO production by macrophages.

In this study, we sought to determine the ability of cloned DuCD40L to stimulate macrophages to produce NO. To obtain a source of DuCD40L, LMH cells were transfected with pcDuCD40L as described in Section 2.2.5 and the supernatant from the LMH cells was then used to treat the chicken macrophage cell line HD11(Lowenthal et al., 1995). NO production by the HD11 cells was then detected using a similar approach to that used to detect NO production by HD11 cells in response to duck IFN-γ (Huang et al., 2001) as
The protein expression in LMH cells transfected with 1.5 µg of pcDuCD40L was tested in Western Blot 48 hr post-transfection as described in Section 2.2.5 and Section 2.2.7. The expression of Flag-tagged DuCD40L protein was probed using anti-Flag monoclonal antibodies followed by goat anti-mouse-HRP antibodies (Table 2-4) as described in Section 2.2.7. The DuCD40L expression was only shown in LMH cells transfected with the pcDuCD40L (Lane 2) but not in LMH cells transfected with the pcDNA3 vector (Lane 1) or with the pFlag vector (Lane 3), indicating that the DNA construct pcDuCD40L is able to express a Flag-tagged DuCD40L protein, ~ 33 kDa in size. The protein blot was stripped and re-probed with rabbit anti-human beta actin polyclonal antibodies followed by goat anti-rabbit-HRP (Table 2-4) as a loading control as described in Section 2.2.7.

Lane 1: Cell lysates of LMH cells transfected with the pcDNA3 vector.

Lane 2: Cell lysate of LMH cells transfected with pcDuCD40L that possesses a flag tag at the C-terminal end.

Lane 3: Cell lysate of LMH cells transfected with a plasmid expressing the Flag epitope (pFlag).
described in Section 2.4.2. The results showed that supernatant collected from LMH cells transfected with pcDuCD40L was able to induce HD11 cells to increase NO production in the culture supernatants at 24 and 48 hr post treatment to levels of around 20 µM and 50 µM respectively. Using supernatants from LMH cells transfected with three different control plasmids, namely the pcDuIL-2 (a pcDNA3 based construct expressing duck IL-2), pcDNA3 vector control and pFlag vector control (Sigma-Aldrich), NO production by HD11 cells was detected at less than 2 µM or was undetectable (Figure 4.5). This finding confirmed the ability of cloned DuCD40L to stimulate macrophages to produce NO. In addition, since we used the supernatants of LMH cells transfected with pcDuCD40L or pcDNA3 control plasmids, the result also indicates that DuCD40L can be secreted as a soluble molecule and shares the same bioactivity as CD40L in mice and rabbit cells (Bingaman et al., 2000; Molero et al., 2005; Tian et al., 1995).

NO production has been reported to be associated with an increase in iNOS gene expression (Hur et al., 1999; Yanagida et al., 2006). In this section we sought to determine if HD11 cells that produced NO in response to DuCD40L also had increased levels of iNOS mRNA. Supernatant from LMH cells transfected with pcDuCD40L tested for the presence of functionally active DuCD40L by incubation of the cell supernatants with HD11 cells. HD11 cells were then harvested 48 hr later with Trizol reagent followed by RNA extraction as described in Section 2.4.1.2. Subsequently, the RNA samples were used to determine the levels of iNOS gene expression in the HD11 cells by qRT-PCR using specific primers designed using the previously reported chicken iNOS gene sequence (Genbank GGU34045) (Table 2-6) as described in Section 2.4.4. The results show that DuCD40L induced iNOS gene expression that was up-regulated ~8 fold compared to the pcDuIL-2, pcDNA3 vector and Flag vector controls (Figure 4.6.). This finding suggests that the production of NO induced by DuCD40L is catalysed by iNOS, which was up-regulated at the transcriptional level.
Figure 4.5. Nitric oxide (NO) production by the chicken macrophage cell line, HD11, treated with DuCD40L

LMH cells were transfected with 1.5 µg of each pcDuCD40L, pcDuIL-2, pcDNA3 and pFlag respectively and maintained at 37°C for 48 hr as described in Section 2.2.5. LMH cell supernatants were analysed for the presence of functional DuCD40L by incubation of the supernatants with the chicken macrophage cell line, HD11, for 24 hr (grey bars) or 48 hr (black bars). Levels of NO produced by the HD11 cells were assayed in the supernatant as described in Section 2.4.2. NO production was observed in HD11 cells co-cultured with supernatant collected from pcDuCD40L transfected LMH. NO production by HD11 cells was measured in the culture supernatants at 24 hr and 48 hr post co-culture to levels of around 20 µM and 50 µM respectively. NO production in the HD11 cells co-cultured with supernatant collected from pcDuIL-2, pcDNA3 and pFlag transfected LMH was at low levels (less than 2 µM) or was undetectable. Analysis confirmed that the ability of the cloned DuCD40L to stimulate NO production by macrophages was statistically significant ($P<0.05$).

*p*-value was calculated using the one way ANOVA by Prism software as described in Section 2.4.3. The *p*-value of <0.05 is considered statistically significant.

* The difference of NO production levels between HD11 cells co-cultured with supernatant collected from pcDuCD40L transfected LMH and with supernatant collected from pcDuIL-2, pcDNA3 and pFlag transfected LMH at 24 hr post co-culture was statistically significant ($p<0.05$).

** The difference of NO production levels between HD11 cells co-cultured with supernatant collected from pcDuCD40L transfected LMH and with supernatant collected from pcDuIL-2, pcDNA3 and pFlag transfected LMH at 48 hr post co-culture was statistically significant ($p<0.05$).
NO level (μm) in the supernatant of HD11 cells

Plasmid constructs used for transfection of LMH cells

pcDNA3  pcDuCD40L  pcDuIL-2  pFlag

24 hr  48 hr
Figure 4.6. The NO production induced by DuCD40L was accompanied by iNOS gene up-regulation.

The qRT-PCR analysis of iNOS gene expression by the chicken macrophage cell line, HD11 as described in Section 2.4.4. The HD11 cells had been co-cultured with cell culture supernatants collected 48 hr after LMH cells were transfected with 1.5 µg of pcDuCD40L, pcDuIL-2, pcDNA3, and pFlag DNA respectively as described in Section 2.2.5. The RNA samples were extracted and cDNA synthesis was performed as described in Sections 2.4.1.2 and 2.4.1.3. Results are presented as the relative fold increase compared to β-actin mRNA. Results from qRT-PCR showed that DuCD40L induced iNOS gene expression was up-regulated ~ 8-fold compared to the pcDuIL-2, pcDNA3, and pFlag vector controls. This finding suggests that the production of NO by DuCD40L is catalysed by iNOS, which was up-regulated at the transcriptional level.

*p-value was calculated using the one way ANOVA by Prism software as described in Section 2.4.3. The p<0.005 is considered statistically significant.

* The difference in iNOS gene expression levels between HD11 cells co-cultured with supernatant collected from pcDuCD40L transfected LMH and with supernatant collected from pcDuIL-2, pcDNA3 and pFlag transfected LMH at 48 hr post co-culture was statistically significant (p<0.05).
Plasmid constructs used for transfection of LMH cells
4.4 *In vivo* Results

In this study, the DuCD40L expression construct was tested to determine: (i) if DNA vaccines expressing DuCD40L enhanced duck humoral immune responses, particularly the anti-DHBs antibodies in ducks, and subsequently reduced the initial DHBV infection in the liver; and (ii) if the protective efficacy of DHBV DNA vaccines was enhanced by DuCD40L by challenging vaccinated ducks with DHBV and assessing levels of DHBV replication and antigen expression in the liver and levels of DHBV DNA in serum.

Two experiments were performed. Experiment I involved the use of 4 Groups of ducks (Groups 4A, 4B, 4C and 4D) and the result showed that the protective efficacy of DHBV DNA vaccine in combination with DuCD40L expression construct was better compared to DHBV DNA vaccine alone group. To obtain more conclusive data, Experiment II was performed under the same conditions as Experiment I but involved the use of 2 Groups of ducks (4E and 4F).

4.4.1 Experiment I

4.4.1.1 Group 4A: The protective efficacy of DHBV DNA vaccines co-administrated with pcDuCD40L on DHBV challenge in 42-day-old ducks

Five DHBV negative ducks in Group 4A were vaccinated i.m. in the thigh muscle, at 14 and 28 days of age, with a mixture of 500 µg of each DHBV DNA vaccine (pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C) co-administrated with 500 µg of pcDuCD40L as described in Section 2.5.2.2. At 42 days of age, two weeks after the second vaccination, all vaccinated ducks from Groups 4A were challenged i.v. with 4.5x10^{10} DHBV genomes.
From the biopsy at day 4 p.c., immuno-staining of DHBsAg revealed that two out of five ducks within Group 4A had a low percentage of DHBsAg-positive hepatocytes (Table 4-2). DHBsAg was detected in the cytoplasm of 0.008% of hepatocytes in duck 4A.1 (0.008%) and in 0.03% of hepatocytes in duck 4A.3 (Figure 4.7 Ai). The remaining three ducks within Group 4A had less than 0.001% DHBsAg-positive hepatocytes on day 4 p.c. However, at the time of a second biopsy on day 14 p.c. and at autopsy on day 31 p.c., all 5/5 Group 4A ducks had undetectable of DHBsAg-positive hepatocytes (<0.001%) (Table 4-2). This indicated that all 5/5 Group 4A ducks had started to clear their DHBV infection from the liver by day 14 p.c.

Liver samples collected at biopsy and autopsy were assayed by qPCR for DHBV DNA. DHBV DNA was detected in all 5/5 Group 4A ducks throughout the study (Figure 4.8). Group 4A ducks had lower levels of mean total DHBV DNA at day 4 p.c. (0.065 copies per cell), at day 14 p.c. (0.032 copies per cell) and at day 31 p.c. (0.013 copies per cell) in comparison to ducks which received DHBV DNA vaccine in Group 4B at day 4 p.c. (11.19 copies per cell), at day 14 p.c. (0.066 copies per cell) and at day 31 p.c. (0.057 copies per cell) (Figure 4.9). These findings suggest that DuCD40L could enhance the DHBV DNA vaccines in inducing specific duck immune responses to control DHBV infection in Group 4A ducks. Larger sample size is needed to obtain statistically significant data.

After DHBV challenge with 4.5x10^{10} DHBV genomes, anti-DHBc antibodies (Figure 4.10) and anti-DHBs antibodies (Figure 4.11) were detected at variable levels in the serum of all 5/5 Group 5A ducks and persisted throughout the study. No anti-DHBc antibodies and anti-DHBs antibodies were detected prior to the DHBV challenge. This indicated that humoral immune responses were activated soon after the onset of DHBV infection.
Legend for Table 4-2

Duck liver tissues were fixed, embedded and sectioned as described in Section 2.5.7. The outcomes of DHBV infection in different treatment groups were determined by the percentage of DHBsAg-positive and DHBcAg-positive hepatocytes on day 4, 14 and 31 p.c.

a Four plasmid DNA constructs were used in this study: pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C, pcDuCD40L, and pcDNA3. Each group was defined by the DNA vaccines given to the ducks.

b Immuno-staining of DHBsAg-positive hepatocytes were performed in EAA-fixed tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in Section 2.7.1.

c Duck liver tissues were collected at 1st biopsy on day 4 and 2nd biopsy on 14 p.c. as described in Section 2.5.5 and at autopsy on day 31 p.c. as described in Section 2.5.6.

d The minimum sensitivity of detection of DHBsAg-positive hepatocytes (0.001%) was based on counting 100,000 hepatocytes in sections of liver tissue collected at biopsy and at autopsy.

e,f,g,h Mean DHBsAg-positive hepatocytes detected in sections of EAA-fixed liver of ducks in Groups 4A, 4B, 4C and 4D at 1st biopsy\(^a\) on day 4 p.c.

i,j,k,l Mean DHBsAg-positive hepatocytes detected in sections of EAA-fixed liver of ducks in Groups 4A, 4B, 4C and 4D at 2nd biopsy\(^b\) on day 14 p.c.

m,n,o,p Mean DHBsAg-positive hepatocytes detected in sections of EAA-fixed liver of ducks in Groups 4A, 4B, 4C and 4D at autopsy\(^c\) on day 31 p.c.

q Liver tissue sections were all observed hepatocytes are positive DHBsAg were recorded as having >95% of DHBsAg-positive hepatocytes.
Table 4-2. The percentage of DHBsAg-positive hepatocytes on day 4, 14 and 31 p.c.

<table>
<thead>
<tr>
<th>Treatment Groups&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Duck No</th>
<th>Duck</th>
<th>% DHBsAg-positive hepatocytes&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 p.c.&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4A</td>
<td>301/302</td>
<td>4A.1</td>
<td>0.008</td>
</tr>
<tr>
<td>(pcDNA3-pre-S/S + pcDNA3-S</td>
<td>303/304</td>
<td>4A.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>+ pcDNA3-C + pcDuCD40L)</td>
<td>305/306</td>
<td>4A.3</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>307/308</td>
<td>4A.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>309/310</td>
<td>4A.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean % DHBsAg-positive hepatocytes</td>
<td>0.019&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>4B</td>
<td>311/312</td>
<td>4B.1</td>
<td>4.1</td>
</tr>
<tr>
<td>(pcDNA3-pre-S/S + pcDNA3-S</td>
<td>313/314</td>
<td>4B.2</td>
<td>0.32</td>
</tr>
<tr>
<td>+ pcDNA3-C)</td>
<td>315/400</td>
<td>4B.3</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>317/318</td>
<td>4B.4</td>
<td>5.26</td>
</tr>
<tr>
<td></td>
<td>319/320</td>
<td>4B.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean % DHBsAg-positive hepatocytes</td>
<td>2.5&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;i&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>4C</td>
<td>321/322</td>
<td>4C.1</td>
<td>3.7</td>
</tr>
<tr>
<td>(pcDuCD40L)</td>
<td>323/324</td>
<td>4C.2</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>325/326</td>
<td>4C.3</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>327/328</td>
<td>4C.4</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>329/330</td>
<td>4C.5</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>396/397</td>
<td>4C.6</td>
<td>5.2</td>
</tr>
<tr>
<td>Mean % DHBsAg-positive hepatocytes</td>
<td>2.88&lt;sup&gt;l&lt;/sup&gt;</td>
<td>63.3&lt;sup&gt;m&lt;/sup&gt;</td>
<td>63.3&lt;sup&gt;n&lt;/sup&gt;</td>
</tr>
<tr>
<td>4D</td>
<td>331/332</td>
<td>4D.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(pcDNA3 plasmid vector</td>
<td>333/334</td>
<td>4D.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>without DHBV challenge)</td>
<td>335/336</td>
<td>4D.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean % DHBsAg-positive hepatocytes</td>
<td>&lt;0.001&lt;sup&gt;o&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;p&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;q&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 4-3. Statistical analysis of the differences in the percentage of DHBsAg-positive hepatocytes on day 4, 14 and 31 p.c.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Day 4 p.c.</th>
<th>Day 14 p.c.</th>
<th>Day 31 p.c.</th>
<th>Mean differences (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 4A vs. 4B</td>
<td>-1.994 (-5.470, 1.482)</td>
<td>0 (-29.925, 29.925)</td>
<td>0 (-37.357, 37.357)</td>
<td></td>
<td>0.2475</td>
</tr>
<tr>
<td></td>
<td><em>p = 0.2475</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4A vs. 4C</td>
<td>-2.868 (-6.196, 0.459)</td>
<td>-63.332 (-91.984, -34.681)</td>
<td>-63.332 (-99.099, -27.565)</td>
<td></td>
<td>0.0878</td>
</tr>
<tr>
<td></td>
<td><em>p = 0.0878</em></td>
<td><em>p &lt; 0.0001</em></td>
<td><em>p = 0.0013</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4B vs. 4C</td>
<td>-0.874 (-4.202, 2.453)</td>
<td>-63.332 (-91.984, -34.681)</td>
<td>-63.332 (-99.099, -27.565)</td>
<td></td>
<td>0.5920</td>
</tr>
<tr>
<td></td>
<td><em>p = 0.5920</em></td>
<td><em>p &lt; 0.0001</em></td>
<td><em>p = 0.0013</em></td>
<td></td>
<td></td>
</tr>
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</table>

*a* Statistical analysis was performed as reported in Section 2.9.

*b* The percentage of DHBsAg-positive hepatocytes was not significantly different in ducks in Groups 4A and 4B at any time-points (*p = 0.2475* and *p = 1*). This indicates DuCD40L did not enhance the protective efficacy of DHBV DNA vaccines.

*c* Differences in the average percentage of DHBsAg-positive hepatocytes between the ducks in Group 4A and 4C as well as between the ducks in Group 4B and 4C were not significantly different at day 4 p.c. (*p = 0.0878* and *p = 0.5920* respectively) but were highly statistically significant at day 14 and 31 p.c. (*p <0.0001*) reflecting that DuCD40L alone is not able to alter the outcome of DHBV infection.
Figure 4.7. The percentage of DHBsAg-positive hepatocytes in each duck from Groups 4A, 4B, 4C and 4D on day 4, 14 and 31 p.c.

Groups of eighteen 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.12.

The percentage of DHBsAg-positive hepatocytes was determined by immuno-staining of EAA-fixed liver tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in the Section 2.7.1. All the sections were photographed using 200 x magnification and sections were counterstained with haematoxylin. Magnification bar = 100 µm. The arrows indicate the DHBsAg (brown staining) in the cytoplasm of infected hepatocytes. The minimum sensitivity of detection of DHBsAg-positive hepatocytes is <0.001%, based on counting 100,000 hepatocytes in sections of liver tissue. Counts were performed using an eyepiece graticule with 250 x 250 µm grid at 200 x magnification.

**Panel A:** Group 4A ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C and pcDuCD40L constructs.

**Panel B:** Group 4B ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C constructs.

**Panel C-I:** Group 4C ducks vaccinated with pcDuCD40L that failed to clear DHBV infection.

**Panel C-II:** Group 4C ducks vaccinated with pcDuCD40L that successfully cleared DHBV infection.

**Panel D:** Group 4D ducks vaccinated with pcDNA3.
Figure 4.8. The levels of total DHBV DNA in the liver of ducks in Groups 4A, 4B, 4C and 4D on days 4, 14 and 31 p.c.

Groups of eighteen 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.12.

Total cellular and virus DNA was extracted from biopsy and autopsy liver tissues as described in Section 2.8.2 and subjected to qPCR analysis for levels of total DHBV DNA using primer set 423-576c (Table 2-6) as described in Section 2.8.5. The limit of detection of the assay shown as the dotted line in Figure 4.8 (A) and (B) is determined by the limit of sensitivity of the qPCR machine which does not accurately detect less than 10 copies of total DHBV DNA per 53,571 cells, or 0.00019 copies of DHBV DNA per cell. In Figure 4.8 (A), the levels of total DHBV DNA in the liver of ducks were grouped by duck number while in Figure 4.8 (B), the levels of total DHBV DNA in the liver of ducks on days 4, 14 and 31 p.c.
Figure 4.8 (A)

DHBV DNA (Copies/liver cell)

Duck Number

4A.1 4A.2 4A.3 4A.4 4A.5 4B.1 4B.2 4B.3 4B.4 4B.5 4C.1 4C.2 4C.3 4C.4 4C.5 4C.6 4D.1 4D.2 4D.3

4 p.c. 14 p.c. 31 p.c.
Figure 4.8 (B) DHBV DNA (Copies/liver cell)
Figure 4.9. The mean levels of total DHBV DNA in the liver of ducks in Groups 4A, 4B, 4C and 4D on days 4, 14 and 31 p.c.

Groups of eighteen 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.12.

Total cellular and virus DNA was extracted from biopsy and autopsy liver tissues as described in Section 2.8.2 and subjected to qPCR analysis for levels of total DHBV DNA using primer set 423-576c (Table 2-6) as described in Section 2.8.5.

The limit of detection of the assay shown as the dotted line in Figure 4.9 is determined by the limit of sensitivity of the qPCR machine which does not accurately detect less than 10 copies of total DHBV DNA per 53,571 cells, or 0.00019 copies of DHBV DNA per liver cell.
Table 4-4. Statistical analysis of the differences of the total DHBV DNA in the liver of ducks in Groups 4A, 4B, and 4C on days 4, 14 and 31 p.c.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean differences (95% CI)</td>
<td>( p ) value</td>
<td>Mean differences (95% CI)</td>
</tr>
<tr>
<td>Group 4A vs. Group 4B</td>
<td>-11.123 (-21.743, -0.503)</td>
<td>( p = 0.0413 )</td>
<td>-0.0339 (-436.137, 436.069)</td>
</tr>
<tr>
<td>Group 4A vs. Group 4C</td>
<td>-6.616 (-16.784, 3.552)</td>
<td>( p = 0.1857 )</td>
<td>-518.729 (-936.265, -101.192)</td>
</tr>
<tr>
<td>Group 4B vs. Group 4C</td>
<td>4.507 (-5.661, 14.675)</td>
<td>( p = 0.3598 )</td>
<td>-518.694 (-936.231, -101.158)</td>
</tr>
</tbody>
</table>

\( a \) Statistical analysis was performed as reported in Section 2.9.

\( b \) The level of total DHBV DNA was highly statistically significant in ducks in Group 4A vs. 4B at day 4 p.c. \( (p = 0.0413) \) but not at day 14 and 31 p.c. \( (p = 0.9999) \). This indicated that DuCD40L could only enhance DHBV DNA vaccines to inducing specific duck immune responses to control DHBV infection at the early stage of DHBV infection but not at the later stage of DHBV infection.

\( c \) The level of total DHBV DNA was not significant in ducks in Group 4A vs 4C \((p = 0.1857)\) and in Group 4B vs 4C \((p = 0.3598)\) at day 4 p.c. but was highly statistically significant at day 14 p.c. \((p = 0.0183)\). The level of total DHBV DNA was then less significant at day 31 p.c. \((p = 0.0951)\) due to the clearance of DHBV infection in 2/6 Group 4C ducks.
Figure 4.10. The anti-DHBc antibodies levels in the sera measured by quantitative ELISA

Groups of eighteen 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.12.

Anti-rDHBc antibodies were measured by qualitative ELISA as described in Section 2.6.3. for each duck from before inoculation (pre-bleed) until day 73 on age (day 31 p.c.). Titres of anti-DHBc antibodies are expressed as the reciprocal of the serum dilution required to achieve an O.D. of 0.4 at 490 nm. The negative cut-off for anti-DHBc-positive samples was determined by the mean O.D. readings of NDS at a dilution of 1 in 1000 plus two standard deviations. As the minimum serum dilution was 1/1000, the minimum titre is 100 (shown as the bottom line in Figure 4.10).

Panel A: Group 4A ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C and pcDuCD40L.
Panel B: Group 4B ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C.
Panel C: Group 4C ducks vaccinated with pcDuCD40L.
Panel D: Group 4D ducks vaccinated with pcDNA3.
Figure 4.11. The anti-DHBs antibodies levels in the sera measured by quantitative ELISA as described in Section 2.6.2. for each duck from before inoculation (pre-bleed) until day 73 on age (day 31 p.c.).

Groups of eighteen 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.12.

Titres of anti-DHBs antibodies are expressed as the reciprocal of the serum dilution required to achieve an O.D. of 0.347 at 490 nm. The negative cut-off for anti-DHBs-positive samples was determined by the mean O.D. readings of NDS at a dilution of 1 in 100 plus two standard deviations. As the minimum serum dilution was 1/100, the minimum titre is 100 (shown as the bottom line in Figure 4.11).

Panel A: Group 4A ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C and pcDuCD40L.

Panel B: Group 4B ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C.

Panel C: Group 4C ducks vaccinated with pcDuCD40L.

Panel D: Group 4D ducks vaccinated with pcDNA3.
The titres of anti-DHBs antibodies in ducks that received DHBV DNA vaccines with pcDuCD40L in Group 4A showed ~10-fold increase compared to Group 4B ducks which received DHBV DNA vaccine and was more than ~15-fold increased compared to the DuCD40L control ducks in Group 4C. The rapid increase in anti-DHBs antibodies following challenge indicated that efficient priming of the immune response had occurred and this also may have restricted the spread of virus from initially infected cells to adjoining cells in the liver. These findings again suggest that DuCD40L had a synergistic effect on the DHBV DNA vaccines that led to an increased in anti-DHBs antibody response.

We expected to see an anti-DHBc antibody response following DHBV challenge in Group 4A ducks compared to other groups (Figure 4.11). The results showed that following DHBV challenge, all the ducks in Groups 4A, 4B and 4C developed high titres of anti-DHBc antibodies rapidly and the levels of antibodies fluctuated throughout this experiment (Figure 4.10). This indicated that efficient humoral immune response was rapidly activated in all these ducks soon after the onset of DHBV infection. However, the levels of anti-DHBc antibodies were not significantly different between these groups. The failure to observe any different between groups could be due to the limitation of our current anti-DHBc antibody ELISA assay condition as maximum anti-DHBc antibodies titre was observed in 5/19 ducks from different groups (Groups 4A, 4B, and 4C). Since anti-DHBc antibodies are non-protective antibodies, they are not involved in the resolution of DHBV infection in any of these ducks and the major role of these anti-DHBc antibodies is that they serve as a marker for current or past DHBV infection.

The serum DHBsAg levels detected at low levels (Range: 0.8-1.035 µg/ml) or below the limit of detection throughout the study (Figure 4.12). This is expected as anti-DHBs antibodies can form immune complexes with the DHBsAg and prevent their detection by ELISA. Similarly, serum DHBV DNA was only detected in duck 4A.2 on day 14 p.c. and 31 p.c. (4 x10³ and 3.3
Figure 4.12. DHBsAg levels in the sera measured by rapid qualitative ELISA.

Groups of eighteen 14-day-old ducks were vaccinated i.m. at thigh muscles twice with various vaccines, at 14 and 28 days of age, using 500 µg of each construct as described in Section 2.5.2.2. At 42 days of age (two weeks after the second vaccination), all vaccinated ducks from Groups 4A, 4B, 4C were challenged with $4.5 \times 10^{10}$ DHBV genomes as described in Section 2.5.1. All Group 4D ducks vaccinated with pcDNA3 plasmid vector were not challenged with DHBV.

The levels of DHBsAg present in the serum were determined by qualitative ELISA using standard curves generated using Pool 8 serum containing 50 µg/mL of DHBsAg (Jilbert et al., 1996) as described in Section 2.6.1. The background level of the DHBsAg ELISA was determined by the mean O.D. readings of NDS plus two standard deviations. The background reading (0.66 µg/mL of DHBsAg) is shown as dotted line in Figure 4.12 and used as the limit of detection for these assays.

Panel A: Group 4A ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C and pcDuCD40L.

Panel B: Group 4B ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C.

Panel C: Group 4C ducks vaccinated with pcDuCD40L.

Panel D: Group 4D ducks vaccinated with pcDNA3.
x $10^3$ copies/ml respectively) and only in duck 4A.4. (5 x $10^3$ copies/ml) at day 28 p.c (Figure 4.13). The detection of serum DHBV DNA in these two ducks might be due to a slight rebound of DHBV infection that was rapidly resolved as subsequently no more serum DHBV DNA was detected for the rest of the study.

In summary, DHBV DNA vaccines co-administered with pcDuCD40L reduced the number of initially DHBV-infected hepatocytes on day 4 p.c. The DuCD40L had a synergistic effect on the production of anti-DHBs antibodies which was boosted when the 42-day-old ducks were challenged with DHBV. The presence of these antibodies may have served to partially neutralise the virus inoculum reducing the percentage of infected cells on day 4 p.c. but may have also bound to newly synthesised virus and prevent its spread in the liver.

4.4.1.2 Group 4B: The protective efficacy of DHBV DNA vaccines on DHBV challenge in 42-day-old ducks

Five DHBV negative ducks in Group 4B were vaccinated i.m. twice at thigh muscle as described in Section 2.5.2.2, at 14 and 28 days of age, with a mixture of 500 µg of each DHBV DNA vaccine (pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C). At 42 days of age, which was two weeks after the second vaccination, all vaccinated ducks from Groups 4B were challenged with 4.5x10^{10} DHBV genomes.

Although it is not statistically significant (Table 4-3), analysis of the liver tissue collected at day 4 p.c. indicated that the number of DHBsAg-positive hepatocytes in Group 4B ducks was increased compared to Group 4A ducks which received DHBV DNA vaccines co-administered with pcDuCD40L (Table 4-2). DHBV infection was found in four out of five Group 4B ducks by immuno-staining of DHBsAg in hepatocytes on day 4 p.c. These four ducks were ducks 4B.1 (4.1%), 4B.2 (0.32%), 4B.3 (0.33%) and 4B.4 (5.26%). The remaining one duck, duck 4B.5 had no detectable DHBsAg-positive hepatocytes (<0.001%).
Figure 4.7 Similar to the Group 4A ducks, hepatocytes expressing DHBsAg were no longer detected in all 5/5 Group 4B ducks at biopsy on day 14 p.c. and at autopsy on day 31 p.c. (Table 4-2). This indicated that all 5/5 Group 4B ducks had cleared DHBV infection in the liver at the end of the experiment. Consistent with the immuno-staining of DHBsAg-positive hepatocytes, the virus replication was also higher in Group 4B with mean total DHBV DNA (11.188 copies per cell) compared to Group 4A (0.065 copies per cell) determined by qPCR in biopsied liver tissues at day 4 p.c. (Figure 4.9).

Analysis of serum revealed that the humoral immune responses in Group 4B were rapid after DHBV challenge, with all 5/5 Group 4B ducks producing anti-DHBc antibodies (Figure 4.10) and anti-DHBs antibodies (Figure 4.11) and persisted throughout the time course. Serum DHBsAg (Figure 4.12) and DHBV DNA (Figure 4.13) were also not detected in four out of five Group 5B ducks that had resolved their DHBV infection by day 14 p.c. The remaining duck from Group 4B, duck 4B.5, had serum DHBsAg detected by quantitative ELISA started from day 14 until day 73. (Figure 4.12). Nonetheless, serum DHBV DNA was not detected by qPCR in duck 4B.5 at any time (Figure 4.13). Since the qPCR assay was more sensitive than ELISA assay, the contradicting results in duck 4B.5 could be due to non-specific binding in the ELISA.

In summary, Group 4B ducks vaccinated with DHBV DNA vaccines (pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C) had restricted initial DHBV infection in the liver on day 4 p.c. and had clear DHBV infection by day 14 p.c.
Figure 4.13. Serum DHBV DNA levels of ducks from Group 4A, 4B, 4C and 4D

Groups of eighteen 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.12.

The levels of DHBV total DNA per mL serum was determined as described in Section 2.8.4 using the primer set 423-576c (Table 2-6). The limit of detection of the assay shown as the dotted line in Figure 4.13 is determined by the limit of sensitivity of the qPCR machine which does not accurately detect less than 10 copies of DHBV total DNA per 4 µL of serum, or 2500 copies of DHBV total DNA per mL of serum.

Panel A: Group 4A ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C and pcDuCD40L.

Panel B: Group 4B ducks vaccinated with pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C.

Panel C: Group 4C ducks vaccinated with pcDuCD40L.

Panel D: Group 4D ducks vaccinated with pcDNA3.
DHBV DNA vaccines, pcDuCD40L, pcDNA3 vector, DHBV challenge
4.4.1.3 Group 4C: The protective efficacy of pcDuCD40L on DHBV challenge in 42-day-old ducks

Six DHBV negative ducks in Group 4C were vaccinated i.m. twice at thigh muscle as described in Section 2.5.2.2, at 14 and 28 days of age, with 500 µg of pcDuCD40L. At 42 days of age, which was two weeks after the second vaccination, all vaccinated ducks from Group 4C were challenged with $4.5 \times 10^{10}$ DHBV genomes.

Following DHBV challenge, all 6/6 Group 4C ducks developed high levels of anti-DHBc antibodies (Figure 4.10) and liver DHBV DNA (Figure 4.8) that persisted throughout the experiment. From the biopsy on day 4 p.c., it was clear that all 6/6 Group 4C ducks had been successfully infected with DHBV as liver tissues from all ducks that received pcDuCD40L had between 0.5 and 5.2% of DHBsAg-positive hepatocytes (Table 4-2).

In this group, four out of six ducks, ducks 4C.1, 4C.2, 4C.3 and 4C.4, were not protected from the development of persistent DHBV infection, and by day 14 p.c., DHBV infection had spread throughout the liver to infect >95% of hepatocytes (Table 4-2; Figure 4.7). Levels of serum DHBV DNA also fluctuated throughout this experiment, in between $7.33 \times 10^4$ and $1.06 \times 10^9$ copies/ml, indicating ongoing active DHBV replication in these four ducks (Figure 4.13).

Based on the previous duck studies performed in our laboratory, widespread DHBV infection in >95% of hepatocytes indicates active virus replication, and serum DHBsAg would be detected in high titres (Foster et al., 2003; Miller et al., 2006, 2008; Feng et al., 2010). Nonetheless, serum DHBsAg was only detected in ducks 4.C1, 4.C3, and 4.C4 on day 31 p.c. (Range: 0.8 to 9 µg/ml) (Figure 4.12). In addition, in duck 4C.1, anti-DHBs antibodies persisted from day 44 to 73 days of age (days 4 – 31 p.c.) (Figure 4.11), indicating simultaneous detection of anti-DHBs antibodies and DHBsAg. Simultaneous detection of
circulating anti-DHBs antibodies and DHBsAg during persistent DHBV infection has been previously reported (Foster et al., 2005; Miller et al., 2006a). Detection of DHBsAg under this condition can be difficult due to the immune complexes formed between anti-DHBs antibodies and circulating serum DHBsAg. Thus, the failure to detect serum DHBsAg in these four ducks could be because vaccination with the pcDuCD40L construct increased the humoral response in these ducks. Following virus challenge, it is likely that the neutralising anti-DHBs antibodies in the serum formed immune complexes with the virus particles, and thus prevented surface antigen detection.

In addition, in concordance with the immuno-staining of DHBsAg in hepatocytes, qPCR analysis of liver DHBV DNA levels also revealed that Group 4C ducks had the highest mean total DHBV DNA at day 4 p.c. (6.68 copies per liver cell), at day 14 p.c. (518.76 copies per liver cell) and at day 31 p.c. (1011 copies per liver cell) compared to Group 4A (DHBV DNA vaccines + pcDuCD40L) and Group 4B (DHBV DNA vaccines) (Figure 4.9). These findings correlate well with the highest mean percentage of DHBsAg-positive hepatocytes found in the Group 4C ducks (Table 4-2).

Interestingly, the remaining two ducks, ducks 4C.5 and duck 4C.6, had <0.001% of DHBsAg-positive hepatocytes at second biopsy on day 14 p.c. and at autopsy on day 31 p.c. (Table 4-2; Figure 4.7). These two ducks were also serum DHBsAg-negative (Figure 4.12) measured by ELISA but had developed serum anti-DHBc antibodies (Figure 4.10). In duck 4C.5, serum DHBV DNA was detected by qPCR only at day 4 p.c. (4.23x10^5 copies/ml) and remained undetectable throughout the time course of study (Figure 4.13). Serum analysis also revealed that duck 4C.5 had developed anti-DHBs antibodies (Figure 4.11), indicating that this duck had been infected but had cleared its DHBV infection. These findings suggest that although vaccination with DuCD40L alone was not able to prevent the early virus spread in the liver; it may play an important role in the virus clearance at later stage of virus infection in ducks.
4C.5 and 4C.6. Nonetheless, it was previously shown that the outcomes of DHBV infection in ducks is age and virus dose dependent, young ducks are more susceptible to DHBV infection and are more likely to develop persistent DHBV infection compared to adult ducks (Jilbert et al., 1998). Thus, the clearance of DHBV infection seen in Group 4C could be either due to these two Group 4C ducks were naturally recovering from acute DHBV infection or that vaccination with DuCD40L improved the immunity of ducks against DHBV infection. To further investigate this, pcDuCD40L control group was repeated in Experiment II and was compared to a pcDNA3 vector control group after virus challenge under the same condition as Experiment I.

In summary, although vaccination with DuCD40L alone was unable to prevent the initial DHBV infection in the liver, it was able to enhance immunity of ducks against DHBV challenge in the absence of DHBV DNA vaccines, resulting the clearance of DHBV infection in two out of six Group 4C ducks by day 14 p.c.

4.4.1.4 Group 4D: The pcDNA3 vector control ducks

Three DHBV negative ducks in Group 4D were vaccinated i.m. twice at thigh muscle as described in Section 2.5.2.2, at 14 and 28 days of age, with 500 µg of pcDNA3 plasmid vector. All three Group 4D ducks were not challenged with DHBV in this experiment.

As expected, all unchallenged ducks in Group 4D (pcDNA3 vector control) did not show DHBV infection in the liver (Figure 4.7). Liver DHBV DNA was not detected by immunostaining of DHBsAg (Table 4-2) and by qPCR (Figure 4.8) throughout the experiment. Serum analysis revealed that DHBsAg (Figure 4.12), anti-DHBc antibodies (Figure 4.10) and serum DHBV DNA (Figure 4.13) were all negative in all three Group 4D ducks during the time course of study. The transient detection of anti-DHBs antibodies in duck 4D.2 could be due to the non-specific binding of ELISA. This is because other infection markers for DHBV
(e.g. anti-DHBc antibodies, liver DHBV DNA) were not detected in this duck. In addition, these serum samples were analysed concurrently with the other ducks, there should not have error in the ELISA.

In summary, the absence of DHBV in the liver and serum detected by immuno-staining, ELISA and qPCR had confirmed all experimental ducks used in this study were DHBV negative.

4.4.2 Experiment II

From Experiment I in this chapter, four out of six Group 4C ducks that vaccinated with pcDuCD40L developed widespread and persistent DHBV infection and the remaining two ducks cleared DHBV infection on day 14 p.c. Due to the lack of an empty vector control group that was challenged with DHBV, it was difficult to determine if the clearance of DHBV infection in these two Group 4C ducks was due to the effect of pcDuCD40L. Therefore, a second experiment involved use of two groups, pcDuCD40L control group (Groups 4E) and pcDNA3 vector control group (Group 4F), five ducks per group, under the same condition as Experiment I.

4.4.2.1 Group 4E: The protective efficacy of pcDuCD40L on DHBV challenge in 42-day-old ducks

Five DHBV negative ducks in Group 4E were vaccinated i.m. twice at thigh muscle as described in Section 2.5.2.2, at 14 and 28 days of age, with 500 µg of pcDuCD40L. At 42 days of age, which was two weeks after the second vaccination, all vaccinated ducks from Groups 4E were challenged with $4.5 \times 10^{10}$ DHBV genomes. In brief, the experimental design in Group 4E was the same with Group 4C and was performed under the same conditions as Experiment I.
As shown in Table 4-5, in Group 4E ducks, after DHBV challenge, the number of DHBV infected hepatocytes at biopsy on day 4 p.c. was within the expected range (Range: 18-26%; Average: 21.4%). This was expected as based on previous studies, inoculation of 42-day-old ducks with 4.5x10^{10} DHBV genomes had lead to DHBV infection in ~30% of hepatocytes in the liver by day 4 p.c (Reaiche, Ph.D. thesis, 2008). However, the average percentage of DHBsAg-positive hepatocytes in Group 4E (21.4%; Table 4-5) was ~7 fold higher than Group 4C (average: 2.88%; Table 4-2) at biopsy on day 4 p.c. All 6/6 Group 4C ducks in Experiment I and 5/5 Group 4E ducks in Experiment II were vaccinated twice with the same DNA constructs expressing DuCD40L, at the same condition before challenge with 4.5x10^{10} DHBV genomes (Figure 4.1). All the experimental ducks in the current studies were inoculated with the same DHBV stock (Pool 8) as described in Section 2.5.1. under the same conditions. Therefore, the difference in percentage of DHBV-infected hepatocytes in Groups 4C and 4E at day 4 p.c. was probably due to different aliquot of DHBV genomes used in the studies or due to the difference in duck immune responses against DHBV infection in individual ducks.

At biopsy on day 14 p.c., in all 5/5 Group 4E ducks, DHBV infection had spread throughout the entire liver, resulting infection of >95% hepatocytes (Figure 4.14; Table 4-5). However, at autopsy on day 31 p.c., only three out of five Group 5E ducks, ducks 5E.1, 5E.2 and 5E.3, had 95% of hepatocytes still DHBsAg-positive (Figure 4.14) and without anti-DHBs antibodies in the serum in majority of the time (Figure 4.15), indicating these ducks failed to clear the infection by end of the experiment. Analysis of serum revealed that serum DHBV DNA levels in these three ducks, ducks 4E.1, 4E.2 and 4E.3, fluctuated between 2.5x10^{4} – 2.68x10^{7} copies/ml (Figure 4.16). It is expected that in persistently DHBV infected ducks, serum DHBV would persist at ~1x10^{5} and 1x10^{9} copies/ml throughout the experiment (Foster et al., 2003; Noordeen, Ph.D. thesis, 2009). Therefore, the findings again indicated that ongoing active DHBV replication occurred in the livers of these three ducks.
Table 4-5. The percentage of DHBsAg-positive hepatocytes in ducks in Groups 4E and 4F on day 4, 14 and 31 p.c.

<table>
<thead>
<tr>
<th>Treatment Groups&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Duck No</th>
<th>Duck</th>
<th>% DHBsAg-positive hepatocytes&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 p.c.&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4E (pcDuCD40L)</td>
<td>475/476</td>
<td>4E.1</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>477/478</td>
<td>4E.2</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>479/480</td>
<td>4E.3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>481/482</td>
<td>4E.4</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>483/484</td>
<td>4E.5</td>
<td>18</td>
</tr>
<tr>
<td>Mean % DHBsAg-positive hepatocytes</td>
<td></td>
<td></td>
<td>21.4&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4F (pcDNA3 plasmid vector)</td>
<td>485/486</td>
<td>4F.1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>487/488</td>
<td>4F.2</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>489/490</td>
<td>4F.3</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>491/492</td>
<td>4F.4</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>493/494</td>
<td>4F.5</td>
<td>16</td>
</tr>
<tr>
<td>Mean % DHBsAg-positive hepatocytes</td>
<td></td>
<td></td>
<td>20&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Duck liver tissues were fixed, embedded and sectioned as described in Section 2.5.7. The outcomes of DHBV infection in different treatment groups were determined by the percentage of DHBsAg-positive and DHBcAg-positive hepatocytes on day 4, 14 and 31 p.c.

<sup>a</sup>Two plasmid DNA constructs were used in this study: pcDuCD40L and pcDNA3..

<sup>b</sup>Immuno-staining of DHBsAg-positive hepatocytes were performed in EAA-fixed tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in Section 2.7.1.

<sup>c</sup>Duck liver tissues were collected at 1<sup>st</sup> biopsy on day 4 and 2<sup>nd</sup> biopsy on day 14 p.c. as described in Section 2.5.5 and at autopsy on day 31 p.c. as described in Section 2.5.6.

<sup>d</sup>Liver tissue sections were all observed hepatocytes are positive DHBsAg were recorded as having >95% of DHBsAg-positive hepatocytes.

<sup>e,f</sup>Mean DHBsAg-positive hepatocytes detected in sections of EAA-fixed liver of ducks in Groups 4E and 4F at 1st biopsy<sup>b</sup> on day 4 p.c.

<sup>g,h</sup>Mean DHBsAg-positive hepatocytes detected in sections of EAA-fixed liver of ducks in Groups 4E and 4F<sup>c</sup> at 2<sup>nd</sup> biopsy<sup>b</sup> on day 14 p.c

<sup>i,j</sup>Mean DHBsAg-positive hepatocytes detected in sections of EAA-fixed liver of ducks in Groups 4E and 4F at autopsy<sup>c</sup> on day 31 p.c.
In contrast, at autopsy on day 31 p.c., the remaining two out of five Group 4E ducks, ducks 4E.4 and 4E.5, showed partial clearance with 36% and 70% of DHBsAg-positive hepatocytes respectively (Table 4-5). Both ducks 4E.4 and 4E.5 also showed patchy/zonal distribution of DHBV-infected hepatocytes in the liver (Figure 4.17), which were previously seen in ducks that were clearing from acute DHBV infection (Reaiche, Ph.D. thesis, 2008; Jilbert et al., 1992), indicating these two ducks were in the process of clearing virus from the livers by day 31 p.c. In these two ducks, hepatocytes in zone 1 near the portal tract (PT) were virus free (DHBsAg-negative) whereas hepatocytes in zone near the central vein (CV) were infected (DHBsAg-negative). Based on the previous work performed in acute WHV-infected woodchuck, patchy/zonal distributed of infected hepatocytes were observed prior to the clearance (Summers et al., 2003). Therefore, it is expected ducks 4E.4 and 4E.5 would have eventually cleared from infection if the experiment were continued beyond 31 p.c. However, whether these two ducks were naturally recovering from acute DHBV infection or it was the DuCD40L that improves the immunity of ducks against DHBV infection and resulted in the clearance of infection remains unknown. As the number of ducks was low and a consistent outcome was not observed within the group, statistical analysis could not be performed in this study.

In addition, anti-DHBs antibodies also co-existed with serum DHBV DNA in ducks 4E.4 and 4E.5 during the course of experiment. Serum analysis revealed that levels of serum DHBV DNA fluctuated between $3.5 \times 10^3$ and $4.7 \times 10^6$ copies/ml (Figure 4.16) whereas anti-DHBs antibodies were first detected at day 56 (14 p.c.), peaked at day 70 (28 p.c.) and dropped at day 73 (31 p.c.) (Figure 4.15). The lower levels of circulating anti-DHBs antibodies in the serum at day 73 (31 p.c.), coinciding with the decrease in number of DHBV-infected hepatocytes in the liver (Table 4-5), presumably indicating infectious DHBV virions or particles were neutralised by these protective antibodies (Foster et al., 2003; Low, Ph.D. thesis, 2012). The results suggested this two Group 5E ducks were in the process of clearing
Figure 4.14. The percentage of DHBsAg-positive hepatocytes in each representative duck from Groups 4E and 4F on days 4, 14 and 31 p.c.

Groups of ten 14-day-old ducks were vaccinated i.m. at thigh muscles twice with various vaccines, at 14 and 28 days of age, using 500 µg of each construct as described in Section 2.5.2.2. At 42 days of age (two weeks after the second vaccination), all vaccinated ducks from Groups 4E and 4F were challenged with $4.5 \times 10^{10}$ DHBV genomes as described in Section 2.5.1.

The percentage of DHBsAg-positive hepatocytes was determined by immuno-staining of EAA-fixed liver tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in the Section 2.7.1. All the sections were photographed using 200 x magnification and sections were counterstained with haematoxylin. Magnification bar = 100 µm. The arrows indicate the DHBsAg-positive hepatocytes (brown staining) in the cytoplasm of infected hepatocytes. The minimum sensitivity of detection of DHBsAg-positive hepatocytes is <0.001%, based on counting 100,000 hepatocytes in sections of liver tissue collected at biopsy and at autopsy. Counts were performed using an eyepiece graticule with 250 x 250 µm grid at 200 x magnification.

Panel A: Group 4E ducks, 4E.1 vaccinated with pcDuCD40L that failed to clear DHBV infection.

Panel B: Group 4E ducks, 4E.4 vaccinated with pcDuCD40L that partially cleared DHBV infection.

Panel C: Group 4F ducks, 4F.1 vaccinated with pcDNA3 plasmid vector.
**Figure 4.15. The anti-DHBs antibodies levels in the sera measured by quantitative ELISA**

Groups of ten 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.14.

Titres of anti-DHBs antibodies are expressed as the reciprocal of the serum dilution required to achieve an O.D. of 0.338 at 490 nm. The negative cut-off for anti-DHBs-positive samples was determined by the mean O.D. readings of NDS at a dilution of 1 in 100 plus two standard deviations. As the minimum serum dilution was 1/100, the minimum titre is 100 (shown as the bottom line in Figure 4.15).

**Panel A**: Group 4E ducks vaccinated with pcDuCD40L.

**Panel B**: Group 4F ducks vaccinated with pcDNA3.
Figure 4.16. Serum DHBV DNA levels of ducks from Groups 4E and 4F

Groups of ten 14-day-old ducks were vaccinated and challenged as described in the legend to Figure 4.14.

The levels of DHBV total DNA per mL of serum was determined by qPCR as described in Section 2.8.4 using the primer set 423-576c (Table 2-6). The limit of detection of the assay shown as the dotted line in Figure 4.13 is determined by the limit of sensitivity of the qPCR machine which does not accurately detect less than 10 copies of DHBV total DNA per 4 µL of serum, or 2500 copies of DHBV total DNA per mL of serum.

Panel A: Group 4E ducks vaccinated with pcDuCD40L.

Panel B: Group 4F ducks vaccinated with pcDNA3.
Figure 4.17. The detection of DHBsAg-positive hepatocytes in duck 4E.5 at day 31 p.c.

The percentage of DHBsAg-positive hepatocytes was determined by immuno-staining of EAA-fixed liver tissues with 1H.1 anti-pre-S/S monoclonal antibodies (Pugh et al., 1995) as described in the Section 2.7.1. All the sections were photographed using 100 x magnification and sections were counterstained with haematoxylin. Magnification bar = 200 µm. Each panel shows a different area with a patchy distribution of DHBsAg-positive hepatocytes. The hepatocytes in zone near the portal tract (PT) were virus free (DHBsAg-negative) whereas hepatocytes in zone near the central vein (CV) were infected (DHBsAg-positive).
the DHBV infection. Again, whether these two ducks were naturally recovering from acute DHBV infection or the DuCD40L enhanced the production of anti-DHBs antibodies and led to the clearance of DHBV infection remains unknown.

In summary, the finding in Group 4E was similar to Group 4C, indicating DuCD40L alone was not efficient in preventing the initial DHBV infection in the liver at day 4 p.c. However, it was able to enhance immunity of ducks against DHBV challenge in the absence of DHBV DNA vaccines, resulting two out of five ducks in Group 4E were in the process of clearing the DHBV infection on day 31 p.c.

**4.4.2.2 Group 4F: The pcDNA3 vector control ducks**

Five DHBV negative ducks in Group 4F were vaccinated i.m. twice at thigh muscle as described in Section 2.5.2.2, at 14 and 28 days of age, with 500 µg of pcDNA3 vector. At 42 days of age, which was two weeks after the second vaccination, all vaccinated ducks from Groups 4F were challenged with 4.5x10^{10} DHBV genomes.

At biopsy on day 4 p.c., liver analysis revealed that DHBsAg-positive hepatocytes were detected in all 5/5 Group 4F ducks (Range: 14 -25%; Average: 20%), similar to Group 4E ducks that vaccinated with pcDuCD40L (Table 4-5; Figure 4.14). At the second biopsy on day 14 p.c., DHBV infection was spread to >95% of hepatocytes and maintained to day 31 p.c. (Table 4-5; Figure 4.14), failing to clear DHBV infection in all 5/5 Group 5F ducks during the course of study. The findings were contrary to Group 4C (pcDuCD40L alone) in Experiment I and Group 4E (pcDuCD40L alone) in Experiment II, in which four out of six ducks and three out of five ducks respectively developed persistent DHBV infection at day 31 p.c. These findings again indicated that DuCD40L alone enhances the immunity of ducks against DHBV challenge. However, it is not known whether the Group 4F ducks would have cleared from the infection if the experiment had not been terminated at day 31 p.c.
Levels of anti-DHBs antibodies and DHBV DNA in the serum were also tested in Group 4F ducks. The anti-DHBs antibodies were not detected in four out five Group 4F ducks during the course of study, except in ducks 4F.1 at day 73 (31 p.c.) with the antibodies just above the level of detection (Figure 4.15). On the other hand, the serum DHBV DNA levels were detectable in all 5/5 Group 4F ducks during the course of experiment, ranging from $1.28 \times 10^5$ – $9.78 \times 10^6$ copies/ml (Figure 4.16). As mentioned above, previous studies showed that in persistently DHBV infected ducks, serum DHBV DNA levels were detected in the range of $\sim 1 \times 10^5$ – $1 \times 10^9$ copies/ml (Foster et al., 2003; Noordeen, Ph.D. Thesis, 2009). This again indicated that DHBV infection was established in all 5/5 Group 4F ducks, which is consistent with the results of the immuno-staining of DHBsAg-positive hepatocytes (Table 4-5).

In summary, all 5/5 pcDNA3 vaccinated ducks in Group 5F were not protected from the initial DHBV infection in the liver at day 4 p.c. and had developed persistent DHBV infection by day 14 p.c.

4.5 Discussion

In this study, we aimed to enhance the efficacy of DHBV DNA vaccines by using genetic adjuvant, a full length DuCD40L DNA construct in the DHBV model. In addition, we modified a previous study (Gares et al., 2006) study at 1) DNA vaccines expressing both DHBV surface and core antigens; 2) Ducks were challenged with high titres of DHBV. This study provided insights into the effectiveness of immune responses against DHBV infection in vaccinated ducks co-administered with full length DuCD40L by testing the levels of initial DHBV infection in the liver as well as serum following virus challenge. To achieve the aim, DuCD40L was subcloned into the pcDNA3 vector and was designated as pcDuCD40L. The success in testing the functionality of the DuCD40L in chicken macrophage HD11 cells showed that HD11 is a suitable system for DuCD40L bioactivity studies despite the DuCD40L only shares ~83% sequence homology to chicken CD40L. The C-terminal region of CD40L is predicted to be
responsible for CD40 binding, the results also demonstrate that insertion of a Flag epitope at the C-terminal end of DuCD40L does not influence the expression or secretion of DuCD40L and does not prevent interaction of CD40L-CD40. To our knowledge, this is the first study of the biological function of DuCD40L by measuring its ability of stimulate macrophages to produce NO.

The ability of DuCD40L to enhance the protective efficacy of DHBV DNA vaccines (Group 4A) was then evaluated by comparing the outcomes in ducks vaccinated with DHBV DNA vaccines only (Group 4B). This was undertaken to determine if co-administration of DuCD40L with DHBV DNA vaccines could reduce the number of initially DHBV-infected hepatocytes at day 4 p.c. following challenge with $4.5 \times 10^{10}$ DHBV genomes as previously investigated in 42-day-old ducks (Reaiche, Ph.D. thesis, 2008). Interestingly, co-administration of DuCD40L with DHBV DNA vaccines in Group 4A ducks resulted in the reduction of DHBV-infected hepatocytes at day 4 p.c. and the enhancement of magnitude of anti-DHBs antibodies response, reaching ~10-fold greater levels than in Group 4B (DHBV DNA vaccines alone) ducks following DHBV challenge.

The increase in magnitude of protective anti-DHBs antibodies response in Group 4A ducks is likely due to the adjuvant effect of DuCD40L on DHBV DNA vaccines and restricted the virus spread in the liver more efficiently. It is known that CD40L on activated CD4+ T cells regulates B lymphocytes differentiation, proliferation and Ig isotype switching via interacting with CD40 expressed on B lymphocytes (Aruffo et al., 1993; Garside et al., 1998). Therefore, when these DHBV DNA vaccines were co-administered with DuCD40L, this exogenous CD40L molecule may act as the naturally occurring CD40L to engage directly with CD40 on activated B lymphocytes and memory B lymphocytes, leading to enhanced antibody production. In addition, interaction between these CD40L molecule and APC might enhance T cells priming and cytokines secretion that promotes activation of B lymphocytes and
induction of memory B lymphocytes to produce antibody responses that helps removing of the antigens. The increase in magnitude of humoral immune responses by co-delivery of DuD40L has been initially demonstrated by a Canadian group in the DHBV model (Gares et al., 2006). Similar to our results, in their study, ducks immunised with DNA vaccines expressing DHBV core antigen plus the functional domain of DuCD40L developed accelerated and enhanced core-specific antibody responses by ~500-fold compared to ducks immunised with DNA vaccines expressing core alone by week 3. However, they did not challenge the co-immunised ducks and thus the core specific CMI in ducks immunised with DNA vaccines expressing DHBV core antigen plus the functional domain of DuCD40L cannot be evaluated. DHBV challenge may have allowed them to determine if induction of CMI directed against DHBV core antigen led to a faster resolution of DHBV infection in ducks immunised with DNA vaccines expressing DHBV core antigen plus the functional domain of DuCD40L. This would allow assessment of the CMI in the absence of neutralising anti-DHBs.

The results also demonstrate that DHBV DNA vaccines expressing pre-S/S, -S and -C in both Groups 4A (with DuCD40L) and 4B (without DuCD40L) eliminated DHBV-infected hepatocytes and prevented the development of widespread DHBV infection by day 14 p.c. The percentage of DHBsAg-positive hepatocytes was not significantly different in ducks in Groups 4A and 4B at any time-point (Table 4-3). This indicates that DuCD40L did not enhance the protective efficacy of DHBV DNA vaccines. DHBV DNA vaccines with or without DuCD40L resulted in the decline of DHBsAg-positive hepatocytes compared to control Group 4F had shown the ability of the DHBV DNA vaccines in simulating specific immune responses against DHBV infection (Table 4-2). It is believed that both DHBV-specific humoral and CMI responses induced by DNA vaccines are responsible for the clearance of infected hepatocytes in this study. The production of neutralising anti-DHBs antibodies induced by DNA vaccines is believed to neutralise circulating viruses and
subsequently prevent the infection in susceptible hepatocytes. The CMI responses induced by DNA vaccines might provide no protection against initial infection but are important in the clearance of infected hepatocytes through cytolytic or non-cytolytic mechanism of CTL. However, due to the lack of duck reagents, CMI responses in duck model cannot be fully elucidated at the present time.

The immune effect of DuCD40L in the absence of DNA vaccine was also tested in this study. Although it did not reach statistical significance ($p = 0.0878$ and $p = 0.5920$ respectively) (Table 4-3), the number of DHBsAg-positive hepatocytes in the Group 4C (pcDuCD40L alone) ducks was higher than the Group 4A (DHBV DNA vaccines + pcDuCD40L) and 4B (DHBV DNA vaccines) ducks at day 4 p.c. (Table 4-2).

In contrast, only four out of six Group 4C ducks developed widespread DHBV infection with > 95% of hepatocytes were DHBsAg-positive on days 14 and 31 p.c. Differences in the average percentage of DHBsAg-positive hepatocytes between the ducks in Group 4A and 4C as well as between the ducks in Group 4B and 4C were highly statistically significant at day 14 and 31 p.c. ($p <0.0001$), reflecting DuCD40L alone is not able on altering the outcome of DHBV infection.

Interestingly, the remaining two Group 4C ducks had no detectable DHBsAg-positive hepatocytes and serum DHBV DNA after day 4 p.c., suggesting these two ducks had resolved their DHBV infection. The ability of DuCD40L in the absence of DNA vaccines (Group 4E) was again evaluated by comparing the outcomes in ducks vaccinated with pcDNA3 vectors only (Group 4F) in Experiment II. The findings further confirm that ducks vaccinated with DuCD40L could enhance immunity of ducks against DHBV challenge in the absence of DNA vaccines, resulting the partial clearance of DHBV infection in 2 Group 4E ducks (Table 4-5; Figure 4.14). This warrants further investigation and greater numbers of ducks need to be examined to determine if this is a statistically significant effect.
The resolution of DHBV infection in the Group 4C ducks in the absence of DHBV DNA vaccine could be due to the enhancement the duck immune responses by DuCD40L. Considering DuCD40L is a modulator of APC function, the expression of DuCD40L by transfected cells at the injection site may explain the enhancement of the duck immune response following DHBV infection. It was proposed that at the site of injection, within the transfected cells, DuCD40L is transcribed and translated to protein. The full-length DuCD40L will be expressed on the transfected cells and it will bind to CD40 expressed on adjacent APC. Upon CD40L-CD40 interaction, the engagement of antigens on MHC molecules on APC results in the induction of APC followed by the maturation and migration of APC to prime T lymphocytes at secondary lymphoid organs. The dying and dead infected cells will also be taken up by APC and processed by the antigen-presenting machinery in order to activate broad anti-virus responses, including the production of anti-DHBs antibodies that bind to DHBsAg to form immune complexes that are efficiently removed from the circulation.

However, as mentioned earlier, the outcome of DHBV infection in ducks is age and virus dose dependent, young ducks are more susceptible to DHBV infection and are more likely to develop persistent DHBV infection compared to adult ducks (Qiao et al., 1990; Vickery and Cossart, 1996; Jilbert et al., 1998; Foster et al., 2005; Miller et al., 2008; Feng et al., 2010; Noordeen et al., 2013). The ducks in both Experiments I and II ducks were 42 days old at the time of virus challenge, indicating these experimental ducks had developed more mature immune system to clear DHBV infection. Thus, it is remains to be elucidated whether ducks vaccinated with pcDuCD40L alone in Group C and Group E were naturally recovering from acute DHBV infection or it was the DuCD40L alone that enhanced the duck immunity against DHBV infection and led to the resolution of DHBV infection.
To validate and determine the adjuvant effect of DuCD40L on DHBV DNA vaccines, in Chapter 5, 14-day-old ducks vaccinated with DHBV DNA vaccines and pcDuCD40L were challenged with $5 \times 10^8$ DHBV genomes, a dose that is 500 times higher than those previously shown to cause persistent DHBV infection in 14-day-old ducks (Jilbert et al., 1998; Foster et al., 2005; Miller et al., 2006b; Feng et al., 2010; Noordeen, Ph.D. thesis, 2009).
Chapter 5: The protective efficacy of a DuCD40L expression construct in combination with DHBV DNA vaccines in 14-day-old ducks

5.1 Introduction and aims

As discussed in Chapter 1, the S-ORF of HBV includes the Pre-S1, Pre-S2 and S domains that allow production of the L, M and S HBV surface proteins respectively (Karayiannis, 2003). The S protein, the smallest of the 3 proteins, comprises ~70% of the HBsAg proteins present in the envelope of HBV virions, while the remaining 30% is comprised of M and L proteins in roughly equal amounts (Heermann et al., 1987; Seeger and Mason, 2000). The HBsAg has an “a” determinant located between AA residues 120 and 150 of the HBV S protein. The HBV “a” determinant provides a dominant epitope for production of neutralising anti-HBs antibodies, a marker for the resolution of HBV infection (Cooreman et al., 2001) and HBV strains with specific mutations in the “a” determinant are not neutralised effectively and are referred to as HBV vaccine escape mutants (Carman et al., 1990; Zuckerman and Zuckerman, 2003).

Currently, recombinant HBV vaccines are either produced in yeast cells (Saccharomyces cerevisiae) or mammalian (Chinese hamster ovary, CHO) cells. The yeast-derived HBV subunit vaccines which contain the HBV S surface protein confer protection against HBV infection in only 80-95% of recipients (Mast et al., 2005). In contrast, the only mammalian cell-derived recombinant HBV vaccine available is GenHevac B®, produced by Pasteur-Mérieux Avantis in France (WHO, 2013). GenHevac B® contains both HBV S surface protein and HBV M (S + Pre-S2) surface protein (Michel et al., 1984).

Studies have also been performed in the DHBV model to test whether the inclusion of the DHBV pre-S/S envelope proteins could improve the efficacy of protective DHBV vaccines.
The S-ORF of DHBV genome encodes two surface antigen proteins, namely the pre-S/S and S proteins. In DHBV, the S protein constitutes about 80% of total DHBV envelope proteins, and the remaining 20% is constituted by pre-S/S protein. DHBV S protein is thus the most abundant protein in the DHBV envelope (Funk et al., 2007). A number of studies have defined neutralising epitopes within the DHBV pre-S/S and S proteins. In Cheung et al., 1989, a library of mABs against DHBV were identified and six of the neutralising mABs were further characterised by epitope mapping. From the competitive binding results, three epitopes recognised by neutralising antibodies were found in pre-S/S domain and only one was found in the S domain. Worth mentioning is that the DHBV S protein does not include the antigenic loop, the so called “a” determinant found in HBsAg because a homologous 150 nt region of the HBV S-ORF is missing from the DHBV S-ORF (Glebe and Urban, 2007).

In Triyatni et al., 1998, DHBV DNA vaccines were used to induce anti-DHBs antibodies in ducks. In the study, six-month-old ducks were vaccinated i.m. with 750 µg DHBV DNA vaccines expressing pre-S/S or S three times, at three weeks intervals. After the third vaccination, high titres of anti-DHBs antibodies that ranged in titre between 1/10,000 and 1/50,000 were detected by ELISA assay in the pre-S/S and S vaccinated ducks. The high titre antibody responses demonstrated the ability of DHBV DNA vaccines to elicit specific humoral responses against DHBV surface proteins in vaccinated ducks. In the same study, despite the presence of approximately equal titres of anti-DHBs antibodies in the pre-S/S and S vaccinated ducks significant differences were observed in both the rate of virus removal from the bloodstream following DHBV challenge, and in the absence or presence of virus replication in the liver of the vaccinated ducks. The study showed that in all four pre-S/S vaccinated ducks, 90% of the DHBV inoculum was removed at similar rates to non-vaccinated ducks between 60 and 90 min p.c., and 10% to 40% of hepatocytes were DHBsAg-positive at 4 days p.c. In contrast, rapid removal of inoculum from the bloodstream (<5 and 15 min p.c.) was observed in three out of four S vaccinated ducks and virus
replication was undetectable in the liver at 4 days p.c. All of the six-month-old ducks then went on to clear their DHBV infection. These findings suggested that DHBV DNA vaccines expressing S protein were more effective at inducing protective immunity than DHBV DNA vaccines expressing pre-S/S. However, this study did not determine if DHBV DNA vaccines expressing pre-S/S or S provided more effective protection against development of persistent DHBV infection. This is because the outcomes of DHBV infection in ducks is age and virus dose dependent; young ducks (e.g. 14-day-old ducks) are more susceptible to DHBV infection and are more likely to develop persistent DHBV infection compared to adult ducks (e.g. 6-month-old) (Jilbert et al., 1998). We have previously shown that, like humans infected with HBV, young ducks are more susceptible to the development of persistent and widespread DHBV infection (Foster et al., 2005; Jilbert et al., 1998; Miller et al., 2008; Thermet et al., 2008; Feng et al., 2010). However, the susceptibility of the newly hatched ducks to the development of persistent and widespread DHBV infection decreased rapidly after hatching, presumably because the older ducks have immune responses that are more effective in clearing the infection (Vickery and Cossart, 1996; Jilbert et al., 1998; Foster et al., 2005). Therefore, the 6-month-old ducks used in the study were able to clear DHBV infection even when inoculated with $2 \times 10^{11}$ DHBV genomes.

From previous work, persistent DHBV infection developed in 14-day-old ducks infected with $5 \times 10^8$ DHBV genomes. This dose of DHBV is 500 times higher than the dose previously shown to cause persistent DHBV infection in 14-day-old ducks (Jilbert et al., 1998; Foster et al., 2005; Miller et al., 2006b). Thus, in Miller et al., 2006b, the ability of DHBV DNA vaccines expressing pre-S/S and S to induce immune responses was tested in 14-day-old ducks, to obtain a more sensitive readout of the protective efficacy of DHBV DNA vaccines expressing pre-S/S and S.
In the study by Miller et al., 2006b, ducks were vaccinated at 4 and 14 days of age with DHBV DNA vaccines encoding the pre-S/S or the S protein. The vaccinated ducks were then challenged with $5 \times 10^8$ DHBV genomes on day 14 of age. This study sought to determine if DHBV DNA vaccines containing either the DHBV pre-S/S or S protein provided protection against DHBV infection in young ducks. The vaccination with DHBV S restricted the initial virus infection in the liver, resulting in an average of 10.7% of DHBsAg-positive hepatocytes on day 4 p.c. compared to 16.5% in unvaccinated control ducks. In contrast, the vaccination with DHBV pre-S/S was not as effective in restricting the initial virus infection in the liver, resulting in an average of 25.06% of DHBsAg-positive hepatocytes on day 4 p.c. compared to 16.5% in unvaccinated control ducks. At the end of the experiment there was no significant difference in the efficacy of the DHBV S and pre-S/S DNA vaccines to modify the outcome of DHBV infection. From the same study, in 14-day-old ducks, only 4 out of the 18 DHBV pre-S/S and S vaccinated ducks (two ducks from each vaccine group) were protected against the development of persistent DHBV infection.

Our studies in Chapter 4 have demonstrated that a DuCD40L expression construct was effective as a genetic adjuvant when delivered at the same time as DHBV DNA vaccines. Vaccination of 6-week-old ducks with a cocktail of DHBV DNA vaccines and a DuCD40L expression construct, followed by challenge with $4.5 \times 10^{10}$ DHBV genomes, led to a significant increase in the anti-DHBs antibody response, reaching ~10-fold greater levels than in ducks vaccinated with DHBV DNA vaccines alone. In summary, these findings suggest that DuCD40L expression construct might have the capacity to enhance the magnitude of vaccine-induced immune responses in our current DHBV model, and DuCD40L warrants further evaluation as an adjuvant for vaccination against DHBV infection.

In this Chapter we describe an extension of the pilot study described in Chapter 4. It has been shown adult ducks are more likely than younger ducks to clear DHBV infection even when
experimentally infected with high dose of DHBV (Vickery and Cossart, 1996; Jilbert et al., 1998; Le Mire et al., 2005; Reaiche et al., 2010). Therefore, to further confirm the findings in Chapter 4, the design of the study was based on the experiments performed by Miller et al., 2006b in 14-day-old ducks. We sought here to determine 1) if the immunity induced by DNA vaccines expressing Pre-S/S more effective than DNA vaccine expressing DHBV S and 2) if a DuCD40L expression construct combined with DNA vaccines expressing DHBV Pre-S/S or S can provide a means to enhance the protective immune responses in DHBV infection and subsequently improve the outcome of DHBV infection.

5.2 Experimental design

In this study, four plasmid DNA constructs were used as previously described in Chapter 4: pcDNA3-pre-S/S, pcDNA3-S, pcDNA3 and pcDuCD40L expression constructs. Thirty one, DHBV negative ducks were divided into 6 Groups, Groups 5A – 5F. The ducks were vaccinated i.m. at 4 days of age, with 250 µg of each DNA construct: Group 5A, pcDNA3-S + pcDuCD40L; Group 5B, pcDNA3-pre-S/S + pcDuCD40L; Group 5C, pcDNA3-S; Group 5D, pcDNA3-pre-S/S; Group 5E, pcDuCD40L alone; Group 5F, pcDNA3 control vector. At 14 days of age, the ducks were vaccinated with a second dose of 250 µg of the same plasmid DNA constructs and were challenged i.v. on the same day with 5x10^8 DHBV genomes (Pool 8) (Jilbert et al., 1996) (Figure 5.1). For each duck a total of 500 µg of DNA was delivered i.m. into the thigh muscle as described previously in Section 2.5.2.2.

All ducks were bled weekly and serum samples were analysed for: (i) the levels of DHBsAg in the serum by rapid qualitative ELISA as described in Section 2.6.1. (ii) the levels of anti-DHBs antibodies in the serum by quantitative ELISA as described in Section 2.6.2. and (iii) serum samples were extracted using the ChargeSwitch gDNA 1 mL serum kit as described in
Section 2.8.1 and served as templates for qPCR assays using primer set 423–567c (Table 2-1) as described in Sections 2.8.3 and 2.8.4.

Liver tissues samples obtained from biopsies (day 4 p.c.) and autopsies (day 21 p.c.) from each duck were used for: (i) immuno-staining of DHBsAg using EAA-fixed duck liver tissues collected on days 4 and 21 p.c. The EAA-fixed tissue and then examined using monoclonal anti-DHBV pre-S/S antibodies, 1H.1 (Pugh et al., 1995) to determine the percentage of virus DHBsAg-positive hepatocytes as described in Section 2.7.1.; and (ii) for immuno-staining of DHBcAg, in formalin-fixed duck liver tissues collected on days 4 and 21 p.c. Sections were treated with an antigen retrieval protocol followed by immuno-staining with polyclonal anti-DHBc antibodies, CQT-2, as described in Section 2.7.2.

5.3 Results

5.3.1 Group 5A: DHBV DNA vaccines expressing DHBV S in combination with a DuCD40L expression construct

Vaccination of ducks with pcDNA3-S and pcDuCD40L prior the DHBV challenge prevented the development of persistent DHBV infection in 6/6 ducks.

The results showed that all 6/6 Groups 5A ducks challenged with 5x10^8 DHBV genomes had no detectable DHBsAg in the serum at any time (Figure 5.2). The serum DHBV DNA levels also remained undetectable in four out of six ducks during the course of experiment, except in ducks 5A.2 and 5A.4 (Figure 5.3 A) where DHBV DNA was detected at 1 time point and within the range of 10^3–10^4 copies/ml.

Anti-DHBs antibodies in the serum were detected in four out of six ducks and remained detectable from day 4 p.c. until day 14 or 21 p.c. (Figure 5.4). The 2 exceptions were: duck
Figure 5.1. The schedule of DNA vaccination, virus challenge, and liver biopsy and autopsy in 14-day-old ducks.

Outline of experiment in Chapter 5. Thirty one, DHBV negative ducks were divided into 6 Groups, Groups 5A – 5F. The ducks were vaccinated i.m. at 4 days of age, with 250 µg of each DNA construct: Group 5A, pcDNA3-S + pcDuCD40L; Group 5B, pcDNA3-pre-S/S + pcDuCD40L; Group 5C, pcDNA3-S; Group 5D, pcDNA3-pre-S/S; Group 5E, pcDuCD40L alone; Groups 5F, pcDNA3 control vector. At day 14 of age, the ducks were vaccinated i.m. with a second dose of 250 µg of the same plasmid DNA constructs and were challenged i.v. on the same day with 5x10^8 DHBV genomes (Pool 8). Liver tissues were collected at two time points after DHBV challenge: at biopsy on day 4 p.c. and at autopsy on day 21 p.c. The experimental groups are summarised in Table 5-1.
Table 5-1. The summary of experimental groups in Chapter 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of ducks</th>
<th>pcDNA3-pre-S/Sa</th>
<th>pcDNA3-Sb</th>
<th>pcDuCD40L</th>
<th>pcDNA3c</th>
<th>DHBV Challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A</td>
<td>6</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5B</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5C</td>
<td>5</td>
<td>-</td>
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<tr>
<td>5D</td>
<td>5</td>
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<td>5F</td>
<td>5</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a Each group was defined by the DHBV DNA vaccines (either pcDNA3-pre-S/S or pcDNA3-S) given to the ducks with or without pcDuCD40L genetic adjuvant.

b Ducks were vaccinated i.m. with 250 µg of pcDNA3-pre-S/S as described in Section 2.5.2.1.

c Ducks were vaccinated i.m. with 250 µg of pcDNA3-S as described in Section 2.5.2.1.

d All thirty one ducks were challenged i.v. at 14 days of age with 5 \times 10^8 DHBV genomes, a dose that is 500 times higher than those previously shown to cause persistent DHBV infection in 14-day-old ducks (Jilbert et al., 1998; Foster et al., 2005; Miller et al., 2006b).
Figure 5.2. DHBsAg levels in the sera measured by rapid qualitative ELISA as described in Section 2.6.1. from each duck before inoculation (pre-bleed) and until day 35 of age (21 p.c.).

Thirty one 4-day-old ducks were vaccinated and challenged as described in the legend to Figure 5.1.

The levels of DHBsAg present in the serum were calculated based on standard curves generated using the Pool 8 serum containing 50 µg/mL of DHBsAg (Jilbert et al., 1996). The background level of the DHBsAg ELISA was determined by the mean O.D. readings of NDS plus two standard deviations. The background reading (3.9 µg/mL of DHBsAg) is shown as dotted line in Figure 5.2 and used as the limit of detection for all these assays.

**Panel A**: Group 5A ducks vaccinated with pcDNA3- S and pcDuCD40L constructs.

**Panel B**: Group 5B ducks vaccinated with pcDNA3- pre-S/S and pcDuCD40L constructs.

**Panel C**: Group 5C ducks vaccinated with pcDNA3-S.

**Panel D**: Group 5D ducks vaccinated with pcDNA3-pre-S/S.

**Panel E**: Group 5E ducks vaccinated with pcDuCD40L.

**Panel F**: Group 5F ducks vaccinated with pcDNA3 plasmid vector.
Figure 5.3. Serum DHBV DNA levels of ducks from Group 5A, 5B, 5C, 5D, 5E and 5F following DHBV challenge by qPCR assay as described in Section 2.8.4

Thirty one 4-day-old ducks were vaccinated and challenged as described in the legend to Figure 5.1.

The levels of DHBV total DNA present per mL serum was determined as described in Section 2.8.4 using the primer set 423-576c (Table 2-7). The limit of detection of the assay shown as the dotted line in Figure 5.3 is determined by the limit of sensitivity of the qPCR machine which does not accurately detect less than 10 copies of DHBV total DNA per 4 µL of serum, or 2500 copies of DHBV total DNA per mL of serum.

Panel A: Group 5A ducks vaccinated with pcDNA3- S and pcDuCD40L constructs.
Panel B: Group 5B ducks vaccinated with pcDNA3- pre-S/S and pcDuCD40L constructs.
Panel C: Group 5C ducks vaccinated with pcDNA3-S.
Panel D: Group 5D ducks vaccinated with pcDNA3-pre-S/S.
Panel E: Group 5E ducks vaccinated with pcDuCD40L.
Panel F: Group 5F ducks vaccinated with pcDNA3.
DHBV DNA vaccines  pcDuCD40L  pcDNA vector  DHBV challenge

**A)**

**B)**

**C)**

**D)**

**E)**

**F)**
Figure 5.4. The anti-DHBs antibody levels in the sera measured by quantitative ELISA as described in Section 2.6.2. from each duck before inoculation (pre-bleed) and until day 35 of age (21 p.c.).

Thirty one 4-day-old ducks were vaccinated and challenged as described in the legend to Figure 5.1.

Titres of anti-DHBs antibodies are expressed as the reciprocal of the serum dilution required to achieve an O.D. of 0.308 at 490 nm. The negative cut-off for anti-DHBs-positive samples was determined by the mean O.D. readings of NDS at a dilution of 1 in 100 plus two standard deviations. As the minimum serum dilution was 1/100, the minimum titre is 100 (shown as the bottom line in Figure 5.4).

Panel A: Group 5A ducks vaccinated with pcDNA3- S and pcDuCD40L constructs.
Panel B: Group 5B ducks vaccinated with pcDNA3- pre-S/S and pcDuCD40L constructs.
Panel C: Group 5C ducks vaccinated with pcDNA3-S.
Panel D: Group 5D ducks vaccinated with pcDNA3-pre-S/S.
Panel E: Group 5E ducks vaccinated with pcDuCD40L.
Panel F: Group 5F ducks vaccinated with pcDNA3 plasmid vector.
DHBV DNA vaccines
pcDuCD40L
pcDNA3 vector
DHBV challenge

Anti-DHBs antibodies

Age (days)
5A.5 which had serum anti-DHBs antibodies detected transiently at day 4 p.c. and duck 5A.6 which had no detectable anti-DHBs antibodies during the course of experiment.

When we compare Group 5A (pcDNA3-S + pcDuCD40L) with Group 5C (pcDNA3-S alone), there were no significant differences in the levels of serum DHBsAg (Figure 5.2), anti-DHBs antibodies (Figure 5.4) in these two groups. Immuno-staining of DHBcAg-positive hepatocytes (Figure 5.5) revealed that establishment of a low-level DHBV infection was found in 2/6 Group 5A (pcDNA3-S + pcDuCD40L) and 3/5 Group 5C (pcDNA3-S) ducks on day 4 p.c. These were ducks 5A.1 (0.007%), 5A.4 (0.016%), ducks 5C.1 (0.02%), 5C.4 (0.007%) and 5C.5 (0.01%), (Table 5-2). However, at autopsy on day 21 p.c., all 6/6 Group 5A and 5/5 Group 5C ducks had undetectable of DHBsAg- and DHBcAg-positive hepatocytes (<0.001%) (Table 5-2), indicating resolution of DHBV infection.

The finding revealed that the DuCD40L expression construct when combined with DNA vaccines expressing DHBV S (Group 5A) showed no significant advantage over DNA vaccines expressing DHBV S alone (Group 5C) in enhancing the protective immune responses in DHBV infection.

### 5.3.2 Group 5B: DHBV DNA vaccines expressing DHBV Pre-S/S in combination with a DuCD40L expression construct

Vaccination of ducks with pcDNA3-pre-S/S and pcDuCD40L prior to DHBV challenge also prevented the development of persistent DHBV infection in 5/5 ducks.

All 5/5 ducks from this group had no detectable serum DHBsAg at any time (Figure 5.2). However, low levels of DHBV DNA were detected in the serum of ducks by qPCR. After DHBV challenge, only duck 5B.1 (4.16 x10³ copies/ml) and duck 5B.3 (1.26 x10⁴ copies/ml) had DHBV DNA detected transiently on day 4 p.c. and 21 p.c. respectively (Figure 5.3B).
When we compared Group 5B (pcDNA3-pre-S/S + pcDuCD40L) with Group 5D (pcDNA3-pre-S/S alone), no significant difference in the levels of serum DHBsAg (Figure 5.2) and DHBV DNA (Figure 5.3) were observed. Similar to Group 5B, all 5/5 Group 5D ducks had no detectable serum DHBsAg and low levels of serum DHBV DNA.

Serum was also tested for the presence of anti-DHBs antibodies. Prior to DHBV challenge, anti-DHBs antibodies were not detected (Figure 5.4 B). The anti-DHBs antibodies in serum were detected in 5/5 ducks in Group 5B and remained detectable from day 4 p.c. until day 14 or 21 p.c. (Figure 5.4 B). When we compared Group 5B (pcDNA3-pre-S/S + pcDuCD40L) with Group 5D (pcDNA3-pre-S/S alone), anti-DHBs antibodies were detected in all Group 5B and 5D ducks at similar levels. The presence of anti-DHBs antibodies indicates resolution of DHBV infection.

Analysis of the liver tissue collected at day 4 p.c. indicated that 1/5 Group 5B and 2/5 Group 5D ducks had low-level DHBV infection detected by immuno-staining of DHBcAg-positive hepatocytes (Table 5-2). These ducks were ducks 5B.1 (0.018%), 5D.3 (0.019%), and 5D.4 (0.003%). However, DHBV-infected hepatocytes were no longer detected (<0.001%) in all 5/5 Group 5B and 5/5 Group 5D ducks on day 21 p.c. (Table 5-2), again indicating resolution of DHBV infection.

The results show that the DuCD40L expression construct combined with DNA vaccines expressing DHBV Pre-S/S (Group 5B) showed no significant advantage over DNA vaccines expressing DHBV Pre-S/S alone (Group 5D) in enhancing the protective immune responses in DHBV infection.
Figure 5.5. The percentage of DHBcAg-positive hepatocytes in a representative duck from Group 5A, 5B, 5C, 5D, 5E and 5F on day 4 and 21 p.c.

Thirty one 4-day-old ducks were vaccinated and challenged as described in the legend to Figure 5.1.

The percentage of DHBcAg-positive hepatocytes in a representative duck from Groups 5A, 5B, 5C and 5D on day 4 and 21 p.c. The percentage of DHBcAg-positive hepatocytes in Group 5E and 5F ducks was available at day 21 p.c. but not at day 4 p.c. due to the fixation problem. The percentage of DHBcAg-positive hepatocytes was determined by immuno-staining of formalin-fixed liver tissues with antigen retrieval using polyclonal anti-DHBc antibodies (CQT-2) as described in the Section 2.7.2. All the sections were photographed using 200 x magnification and sections were counterstained with haematoxylin. Magnification bar = 100 µm. The arrows indicate the DHBcAg-positive hepatocytes (brown staining) in the cytoplasm of infected hepatocytes. The minimum sensitivity of detection of DHBcAg-positive hepatocytes is <0.001%. Counts were performed using an eyepiece graticule with 250 x 250 µm grid at 200 x magnification.

Panel A: Group 5A duck, 5A.4 vaccinated with pcDNA3- S and pcDuCD40L constructs.
Panel B: Group 5B duck, 5B.1 vaccinated with pcDNA3-pre-S/S and pcDuCD40L constructs.
Panel C: Group 5C duck, 5C.1 vaccinated with pcDNA3- S.
Panel D: Group 5D duck, 5D.4 vaccinated with pcDNA3- pre-S/S.
Panel E: Group 5E duck, 5E.1 vaccinated with pcDuCD40L that failed to clear DHBV infection.
Panel F: Group 5E duck, 5E.3 vaccinated with pcDuCD40L in the process of DHBV clearance.
Panel G: Group 5F duck, 5E.1 vaccinated with pcDNA3.
Legend for Table 5-2

Duck liver tissues were fixed, embedded and sectioned as described in Section 2.5.7. The outcomes of DHBV infection in different treatment groups were assessed by determining the percentage of DHBsAg-positive and DHBcAg-positive hepatocytes on day 4 and 21 p.c.

a Four plasmid DNA constructs were used in this study: pcDNA3-pre-S/S, pcDNA3-S, pcDuCD40L, and pcDNA3. Each group was defined by the DNA vaccines given to the ducks.

b Immuno-staining of DHBsAg-positive hepatocytes was performed in EAA-fixed tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in Section 2.7.1.

c Immuno-staining of DHBcAg-positive hepatocytes was performed in antigen retrieval treated formalin-fixed tissues with anti-DHBc polyclonal antibodies (CQT-2) as described in Section 2.7.2.

d Duck liver tissues were collected at biopsy on day 4 p.c. and at autopsy on day 21 p.c. as described in Sections 2.5.5 and 2.5.6 respectively.

e The minimum sensitivity of detection of DHBsAg-positive hepatocytes (0.001%) was based on counting 100,000 hepatocytes.

f Liver tissue sections were all observed hepatocytes are DHBsAg-positive were recorded as having >95% of DHBsAg-positive hepatocytes.

g The percentage of DHBV positive hepatocytes was not determined (ND).
Table 5-2. Detection of DHBsAg- and DHBcAg-positive hepatocytes.

<table>
<thead>
<tr>
<th>Groups*</th>
<th>Duck No</th>
<th>Duck</th>
<th>% DHBsAg-positive hepatocytes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% DHBcAg-positive hepatocytes&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>4 p.c.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21 p.c.&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>5A</td>
<td>352/353</td>
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<td>0.003</td>
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<tr>
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<td>346/347</td>
<td>5E.4</td>
<td>0.002</td>
<td>&gt;95</td>
</tr>
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<td>348/349</td>
<td>5E.5</td>
<td>0.009</td>
<td>&gt;95</td>
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<td>&gt;95</td>
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<td>63/100</td>
<td>5F.5</td>
<td>0.019</td>
<td>&gt;95</td>
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5.3.3 Group 5C: DHBV DNA vaccines expressing DHBV S

All 5/5 ducks from this group also had no detectable DHBsAg in the serum at any time (Figure 5.2 C) but developed detectable anti-DHBs antibodies in serum and remained detectable from day 4 p.c. until day 14 or 21 p.c. (Figure 5.4 C). The titres of anti-DHBs antibodies fluctuated during the course of experiment and were not detected prior to the DHBV challenge. Again, the absence of serum DHBsAg but presence of anti-DHBs antibodies in the serum suggests that all 5/5 Group 5C ducks had resolved DHBV infection.

The qPCR analysis of serum detected small spikes of serum DHBV DNA in three of five ducks (ducks 5C.1, 5C.2 and 5C.4) and levels were within the range of 2.70 - 9.45 x 10³ copies/ml (Figure 5.3 C). The serum DHBV DNA levels were consistent with the expected serum DHBV DNA levels in ducks resolving from DHBV infection in previously reported age-matched ducks (Noordeen, Ph.D. thesis, 2009).

When we compared Group 5C (pcDNA3-S alone) with Group 5D (pcDNA3-Pre-S/S alone), immuno-staining of DHBcAg (Figure 5.5) revealed that low-level DHBV infection was detected in 3/5 Group 5C and 2/5 Group 5D ducks at biopsy on day 4 p.c. (Table 5-2), which were ducks 5C.1 (0.02%), 5C.4 (0.007%), 5C.5 (0.01%), 5D.3 (0.019%), and 5D.4 (0.003%). However, at autopsy on day 21 p.c., DHBcAg-positive hepatocytes were no longer detected in all 5/5 Group 5C ducks by immuno-staining (Table 5-2). Similarly, ducks vaccinated with pcDNA3-pre-S/S (Group 5D) vaccine had undetectable of DHBsAg- and DHBcAg-positive hepatocytes (<0.001%) at day 21 p.c. (Table 5-2), suggesting that all Group 5C and 5D ducks had cleared DHBV infection. The finding suggests that the immunity induced by DNA vaccines expressing Pre-S/S had similar protective efficacy when compared to DNA vaccine expressing DHBV S.
In contrast, vaccination with the pcDNA3-S vaccine (Group 5C) was effective in preventing widespread DHBV infection when compared to the ducks that were vaccinated with the pcDNA3 vector (Group 5F). DHBsAg-positive hepatocytes were detected in all 5/5 Group 5F (pcDNA3 vector) ducks (range: 0.002-0.16%) and DHBV infection had spread throughout the entire liver, resulting in infection of >95% of hepatocytes at autopsy on day 21 p.c. (Figure 5.6; Table 5-2).

5.3.4 Group 5D: DHBV DNA vaccines expressing DHBV Pre-S/S

DHBV DNA vaccines expressing DHBV Pre-S/S did not prevent initial DHBV infection of the liver of the Group 5D ducks, 5D.3 (0.019%) and 5D.4 (0.003%) at day 4 p.c. detected by immuno-staining of DHBcAg-positive hepatocytes. However, at day 21 p.c. no DHBcAg-positive hepatocytes were detected and these two ducks had cleared DHBV infection. The remaining 3/5 ducks had <0.001% of DHBsAg and DHBcAg-positive hepatocytes at biopsy on day 4 p.c. and at autopsy on day 21 p.c. (Table 5-2).

All 5/5 ducks had no detectable DHBsAg in the serum at any time (Figure 5.2 D). However, low levels of serum DHBV DNA were detected in all 5/5 ducks with variable levels (within the range of 2.9x10^3 - 2.4x10^4 copies/ml (Figure 5.3 D), both between individual animals and within individuals over time.

Anti-DHBs antibodies were seen in all 5/5 ducks (Figure 5.4 D). Two ducks, 5D.4 and 5D.5, had serum anti-DHBs antibodies detected transiently after DHBV challenge. The remaining 3/5 ducks had anti-DHBs antibodies in the serum after DHBV challenge which remained detectable at autopsy on day 21 p.c. The presence of anti-DHBs antibodies without the serum DHBsAg provides further evidence that these ducks had recovered from DHBV infection.
Again, vaccination with the pcDNA3-Pre-S/S vaccine (Group 5D) was effective in preventing widespread DHBV infection when compared to the ducks vaccinated with the pcDNA3 vector (Group 5F) (Table 5-2). All 5/5 Group 5F ducks progressed to widespread DHBV infection with >95% of hepatocytes staining positive for DHBsAg at autopsy on day 21 p.c. (Figure 5.6; Table 5-2).

In summary, vaccination of ducks with pcDNA3-pre-S/S prior the DHBV challenge prevented the development of widespread DHBV infection.

5.3.5 Group 5E: DuCD40L expression construct alone

In this group, we wished to determine if vaccination with DuCD40L alone prior to the DHBV challenge could alter the DHBV infection outcome in 14-day-old ducks. All 5/5 DuCD40L vaccinated ducks in Group 5E had similar numbers of DHBsAg-positive hepatocytes at biopsy on day 4 p.c. (range: 0.002-0.009%) compared with the pcDNA3 vector control ducks (Group 5F) challenged with the same dose (range: 0.002-0.16%) (Table 5-2).

Analysis of liver tissue collected at autopsy on day 21 p.c. showed that three out of five DuCD40L vaccinated ducks (ducks 5E.1, 5E.4 and 5E.5) developed widespread DHBV infection with >95% of hepatocytes were DHBsAg-positive, failing to clear the infection by the end of the experiment (Table 5-2, Figure 5.6). The remaining two Group 5E ducks, ducks 5E.2 and 5E.3, had <0.001 and 38.5% DHBsAg-positive hepatocytes respectively at day 21 p.c. (Table 5-2). This indicated that duck 5E.2 had cleared the infection before the autopsy at day 21 p.c. The outcome of DHBV infection in duck 5E.3 is unknown. The duck may have been in the process of clearing infected hepatocytes. In contrast, all five of the pcDNA3 vector control ducks (Group 5F) developed widespread DHBV infection with >95% of hepatocytes staining DHBsAg-positive at autopsy (Table 5-2).
Figure 5.6. The percentage of DHBsAg-positive hepatocytes in a representative duck from Groups 5A, 5B, 5C, 5D, 5E and 5F on day 4 and 21 p.c.

Thirty one 4-day-old ducks were vaccinated and challenged as described in the legend to Figure 5.1.

The percentage of DHBsAg-positive hepatocytes was determined by immuno-staining of EAA-fixed liver tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in the Section 2.7.1. All the sections were photographed using 200 x magnification and sections were counterstained with haematoxylin. Magnification bar = 100 µm. The arrows indicate the DHBsAg-positive hepatocytes (brown staining) in the cytoplasm of infected hepatocytes. The minimum sensitivity of detection of DHBsAg-positive hepatocytes is <0.001%, based on counting 100,000 hepatocytes in sections of liver tissue. Counts were performed using an eyepiece graticule with 250 x 250 µm grid at 200 x magnification.

Panel A: Group 5A duck, 5A.1 vaccinated with pcDNA3-pre-S/S and pcDuCD40L constructs.

Panel B: Group 5B duck, 5B.1 vaccinated with pcDNA3-S and pcDuCD40L constructs.

Panel C: Group 5C duck, 5C.1 vaccinated with pcDNA3-pre-S/S.

Panel D: Group 5D duck, 5D.1 vaccinated with pcDNA3-S.

Panel E: Group 5E duck, 5E.1 vaccinated with pcDuCD40L that failed to clear DHBV infection.

Panel F: Group 5E duck, 5E.3 vaccinated with pcDuCD40L that in the process of DHBV clearance.

Panel G: Group 5F duck, 6F.3 vaccinated with pcDNA3.
The percentage of DHBcAg-positive hepatocytes at day 4 p.c. was not able to be determined in this group. This could be due to the poor conservation of tissue section or to a fixation problem. However DHBcAg detection was performed on the autopsy liver samples collected on day 21 p.c. In agreement with the results from immuno-staining of DHBsAg, <0.001% and 34% of DHBcAg-positive hepatocytes were detected in 5E.2 and 5E.3 ducks respectively whereas the remaining three ducks developed DHBV infection in >95% of hepatocytes at autopsy on day 21 p.c. (Table 5-2).

All the ducks in Groups 5E challenged with 5x10⁸ DHBV genomes had no detectable DHBsAg in the serum at any time except duck 5E.5, which had developed widespread DHBV infection at autopsy. This duck had DHBsAg detected in the serum on the last day of experiment on day 21 p.c. and the DHBsAg titres were slightly above the cut-off level of the ELISA assay, measured at 4.106 µg/ml (Figure 5.2 E).

Serum DHBV DNA levels revealed that after DHBV challenge, all ducks had DHBV DNA detected in the serum (range 10⁴–10⁵ copies/ml) on the day 4 p.c. except duck 5E.5. However, from day 7 p.c. onward, serum DHBV DNA, was constantly detectable by qPCR assay in ducks 5E.1, 5E.4 and 5E.5 and within the range of 10⁴-10⁸ copies/ml during the course of experiment, indicating ongoing active virus replication in the liver (Figure 5.3 E). In contrast, duck 5E.2 had detectable DHBV DNA in the serum on days 4 and 7 p.c. (6.25 x 10⁴ and 1.26x 10⁵ copies/ml respectively) but the serum DHBV DNA levels had later fallen to undetectable levels, indicating the resolution of DHBV infection. For duck 5E.3, serum DHBV DNA was first detected on day 7 p.c. (6.35 x 10³ copies/ml) and became undetectable on day 14 p.c. but rebounded on day 21 p.c. (7.41 x 10⁵ copies/ml) (Figure 5.3 E).
The presence of anti-DHBs antibodies were also detected in ducks 5E.2, 5E.3 and 5E.4 (Figure 5.4 E). In duck 5E.2, anti-DHBs antibodies were consistently detected from day 7 p.c. and were maintained until autopsy (day 21 p.c.), indicating resolution of DHBV infection. In contrast, duck 5E.3 and 5E.4 had serum anti-DHBs antibodies that were only transiently detected in the serum. In duck 5E.3, transient detection of anti-DHBs antibodies in serum on day 4 and 14 p.c., resulted in ~34-38% of hepatocytes were DHBV-infected (Table 5-2) at day 21 p.c. In duck 5E.4 that developed widespread DHBV infection, serum anti-DHBs antibodies were detected only transiently on day 14 p.c., again suggesting the failure of duck immune response to clear the infection. The detection of anti-DHBs antibodies in duck 5E.4 was not unexpected as anti-DHBs antibodies can also be detected at low levels in the sera of congenitally and experimentally DHBV-infected ducks with persistent DHBV infection (Miller et al., 2004).

In summary, only 3/5 Group 5E ducks that received pcDuCD40L prior to the DHBV challenge had developed persistent DHBV infection compared to all 5/5 pcDNA3 vector control ducks in Group 5F. Based on these preliminary results and it is possible that delivery of the genetic adjuvant DuCD40L may be able to enhance immunity of ducks against DHBV challenge. However, further studies are required with larger numbers of animals to validate this observation with statistically significant results as DuCD40L did not enhance the protective efficacy of DNA vaccines expressing DHBV Pre-S/S or S in Group 5A and 5B respectively.

5.3.6 Group 5F: The pcDNA3 vector alone

In liver tissue collected at biopsy on day 4 p.c., immuno-staining of DHBsAg present in the cytoplasm of DHBV-infected hepatocytes (Figure 5.6) revealed that DHBV infection was detected 5/5 ducks in 0.002-0.16% of hepatocytes (Table 5-2). At autopsy on day 21 p.c.,
DHBV infection had spread throughout the entire liver in all 5/5 ducks, resulting in infection of >95% of hepatocytes (Figure 5.6; Table 5-2).

The number of DHBcAg-positive hepatocytes on day 4 p.c. was again not able to be determined due to the poor conservation of tissue sections and a fixation problem. At autopsy on day 21 p.c., the results were consistent with the finding of immuno-staining of DHBsAg-positive hepatocytes, all 5/5 Group 5F ducks showed widespread DHBV infection with >95% of hepatocytes were DHBcAg-positive, failing to clear the infection by end of the experiment (Table 5-2, Figure 5.5).

The serum DHBsAg was only detected in ducks 5F.3 and 5F.4. In ducks 5F.3, serum DHBsAg was only detected on day 14 p.c., and the level was ~23 µg/mL (Figure 5.2 F). In duck 5F.4, during the later course of the experiment, serum DHBsAg was detected on day 7 and 21 p.c., at 7.8 µg/mL and 12 µg/mL respectively (Figure 5.2 F). From the studies performed by Miller et al., 2006b, all 14-day-old pcDNA1.1 Amp vector (Invitrogen) vaccinated ducks challenged with 5x10⁸ DHBV genomes had readily detectable serum DHBsAg started on day 7 p.c. and maintained until autopsy. The failure to detect serum DHBsAg in majority of Group 5E ducks could be due to the immune complex production with serum anti-surface antibody blocking the detection by ELISA (Tohidi-Esfahani et al., 2010).

In all 5/5 ducks, DHBV DNA was detectable in the serum and within the range of 10⁴ –10⁶ copies/ml on day 4 p.c. Then the DHBV DNA levels fluctuated but remained within the range of 10⁴-10⁹ copies per ml during the course of experiment (Figure 5.3 F). The high levels of persistent DHBV DNA in the serum reflect ongoing active virus replication in the liver, consistent with the results of immuno-staining for DHBsAg- and DHBcAg-positive hepatocytes (Table 5-2).
All 5/5 ducks that showed widespread DHBV infection in the liver at day 21 p.c., had no detectable anti-DHBs antibodies throughout the course of experiment (Figure 5.4 F). This is expected as the detection of anti-DHBs antibodies is commonly used as a marker of resolution of DHBV infection, and not detected in persistently DHBV-infected ducks (Jilbert and Kotlarski, 2000; Kuroki et al., 1995; Vickery et al., 1989).

In Summary, all 5/5 Group 5F ducks that received the pcDNA3 vector prior to the DHBV challenge failed to clear DHBV infection and developed persistent DHBV infection.

5.4 Discussion

It has been shown that young ducklings are exquisitely susceptible to the development of persistent DHBV infection (Jilbert et al., 1996; Vickery and Cossart, 1996; Tohidi-Esfahani et al., 2010). From previous work, once 14-day-old ducks are infected with 5 x10^8 DHBV genomes, virus infection spreads throughout the liver and they remain persistently DHBV infected (Jilbert et al., 1998; Foster et al., 2005; Miller et al., 2006b; Feng et al., 2010). In contrast, in adult ducks (e.g. 6-week-old), there is progressive loss of susceptibility to DHBV infection, so that these ducks are able to clear DHBV infection even when experimentally infected with high dose of DHBV (Jilbert et al., 1998; Le Mire et al., 2005; Reaiche et al., 2010). In addition, as the neutralising anti-DHBs antibodies could limit the liver infection by preventing secondary cycles of infection, the acute DHBV infection outcome in adult ducks could also be due to their ability to develop more rapid neutralising anti-DHBs antibody responses compared with young ducklings (Zhang and Summers, 2004).

Our studies in Chapter 4 have demonstrated that i.m. vaccination of 6-week-old ducks with a cocktail of DHBV DNA vaccines and the DuCD40L expression construct, followed by challenge with 4.5x10^{10} DHBV genomes, led to significant increases in the anti-DHBs
antibody response, reaching ~10-fold greater levels than in ducks vaccinated with DHBV DNA vaccines alone. However, as mentioned earlier, the ducks in Chapter 4 were 6-week-old at the time of virus challenge, suggesting that these experimental ducks had developed a more mature immune system allowing them to clear DHBV infection. Thus, it is remains to be elucidated whether these ducks were naturally recovering from acute DHBV infection or it was the DuCD40L expression construct that enhanced the immunity against DHBV infection.

Therefore, to ascertain the effect of DuCD40L expression construct on DHBV DNA vaccines, in this Chapter, the study aimed to compare the DHBV infection outcomes in 6-week-old ducks (Chapter 4) and 14-day-old ducks to determine if 1) the DuCD40L expression construct co-administrated with DHBV DNA vaccines could enhance the magnitude of anti-DHBs antibody responses and 2) if DuCD40L expression construct combined with DNA vaccines expressing DHBV Pre-S/S or S can provide a means to enhance the protective immune responses in DHBV infection and subsequently alter the DHBV infection outcomes in our well-established duck model.

In this study, after two doses of vaccination, similar levels of anti-DHBs antibodies were observed in all Groups 5A (pcDNA3-S + pcDuCD40L), 5B (pcDNA3-pre-S/S + pcDuCD40L), 5C (pcDNA3-S alone) and 5D (pcDNA3-pre-S/S alone) ducks, with titres of anti-DHBs antibodies that ranged between 100 and 1000 (Figure 5.4). These findings suggest that co-administration of DHBV DNA vaccines with a DuCD40L expression construct in Group 5A and 5B ducks did not enhance the magnitude of the anti-DHBs antibody response in comparison to Group 5C and 5D ducks that were vaccinated with DHBV DNA vaccines alone (Figure 5.4). This finding is at odds with results in Chapter 4, following DHBV challenge, 6-week-old ducks vaccinated with DHBV DNA vaccine and DuCD40L (Group 4A) resulted in an enhancement of the magnitude of the anti-DHBs antibody response, reaching ~ 10-fold greater levels than in ducks vaccinated with DHBV DNA vaccines only.
(Group 4B) (Figure 4.11). This has made the objective in this study difficult to assess. We cannot tell whether there was synergistic effect between DuCD40L and the DHBV DNA vaccines or not.

There are two possible reasons for this inconsistency. In young ducklings, the ability to induce rapid humoral immune responses is not developed in the first few weeks of life (Fukuda et al., 1987). Due to this immunological immaturity, several days are required to develop humoral immune responses in these young ducklings (Vickery et al., 1989). In contrast, by 6 weeks of age, the ability of these ducks in inducing humoral immune responses has been amply demonstrated (Mason et al., 1983; Omata et al., 1984; Le Mire et al., 2005; Reaiche et al., 2010). Therefore, the relative immaturity of the immune system in 14-day-old ducks compared to 6-week-old ducks at the time of virus challenge, could affect the ability of the vaccinated ducks to induce higher anti-DHBs antibody titres following virus challenge. In addition, the antibody response induced by DNA vaccine is dependent on various factors such as the plasmid DNA dose and animal body weight (Lai and Bennett, 1998). Here, the dose of DHBV DNA vaccines given to 6-week-old ducks (500 µg of each DNA vaccine construct per dose) was doubled compared to the 14-day-old ducks (250 µg of each DNA vaccine construct per dose). This might increase the expression of the cloned antigens in the context of MHC complex at the surface of transfected cells and induce the production of anti-DHBs antibodies in the 6-week-old ducks more efficiently following DHBV challenge.

In Gares et al., 2006, ducks immunised with DNA vaccines expressing a chimera of DuCD40L fused to a truncated form of DHBV core antigen (tcore-DuCD154) exhibited accelerated and stronger core antigen-specific humoral immune responses compared to the control group of ducks immunised with plasmid DNA expressing DHBV core antigen alone. Ideally, serum anti-DHBc antibody responses should be determined to investigate whether the DuCD40L expression construct enhanced DNA vaccine responses in ducks, resulting in
stronger anti-DHBc antibody responses compared to ducks vaccinated with DHBV DNA vaccines alone. Unfortunately, levels of serum anti-DHBc antibodies were not able to be measured using an ELISA assay in this study due to high background reading (data not shown).

Another somewhat unexpected observation in this protective DNA vaccine study in 14-day-old ducks was that following DHBV challenge, no significant differences in the percentage of DHBV-infected hepatocytes (Table 5-2) or anti-DHBs antibody titres (Figure 5.4) were observed between ducks receiving DHBV DNA vaccines expressing either pre-S/S or S antigens alone (Groups 5D and 5C respectively). All 10 Group 5C and 5D ducks had cleared their DHBV infection by day 21 p.c. (Table 5-2).

These results contradict the findings of Miller et al., 2006b, where only 4 out of the 18 pre-S/S and S vaccinated ducks (two ducks from each vaccine) challenged with 5x10⁸ DHBV genomes were protected against the development of persistent DHBV infection, compared to 10 out of 10 pre-S/S and S vaccinated ducks in Groups 5C and 5D of this study.

From the same study by Miller et al., 2006b, 14-day-old ducks vaccinated with DHBV DNA vaccine expressing pre-S/S and S antigens had protective immune responses that reduced the number of initial DHBV-infected hepatocytes at day 4 p.c. and prevented the development of persistent DHBV infection in a virus dose-dependent manner. In the study, statistically significant protection was observed only at a challenge dose of 5x10⁷ DHBV genomes but not at 5x10⁸ DHBV genomes, where ducks were not protected from the development of persistent DHBV infection (Miller et al., 2006b).

Therefore, we proposed that the inconsistency of the results obtained from Miller et al., 2006b and this study could be due to a decline in the infectivity of the inoculum used in this study.
Based on previous studies performed in our laboratory, 14-day-old ducks challenged with 5x10^6 DHBV genomes would have approximately 2-40% of DHBV-infected hepatocytes on day 4 p.c. (Miller et al., 2006b, 2008; Noordeen Ph.D. Thesis, 2009). In this current study, 14-day-old vector-vaccinated ducks challenged with same dose of virus had 0.002 to 0.16% of DHBsAg-positive hepatocytes (Table 5-2), a level 1000-fold lower than could be expected from the original inoculum. It was possible that a decline in the infectivity of the inoculum could provide opportunity for vaccine-induced immune responses to reduce the number of initial infected hepatocytes in the livers on day 4 p.c. and resulted in the clearance of the virus infection in these ducks by day 21 p.c.

Although a decline in the infectivity of the inoculum used in this current study was possible, persistent DHBV infection was successfully established in 5 out of 5 vector-vaccinated ducks in Group 5F, indicating the virus challenge dose given to the ducks was still higher than those known to result in persistent DHBV infection in non-vaccinated 14-day-old ducks. To get a conclusive result, the current experiment needs to be repeated and it is advisable to determine the new infectivity titres (ID_{50}) of the current inoculum in ducks before repeating the experiment.

Nonetheless, as persistent DHBV infection was successfully established in all 5 vector-vaccinated ducks in Group 5F, the results in this study again demonstrate the ability of DHBV DNA vaccines expressing pre-S/S or S antigens to induce protective immune responses following DHBV challenge. As summarised in Table 5-2, our results revealed that the presence of low but detectable amount of DHBcAg-positive hepatocytes (0.003-0.02%) within the livers of 7 out of the 21 Groups 5A-5D ducks at day 4 p.c. was later undetectable (<0.001%) at day 21 p.c.
Notably, low levels of early virus replication were detected in the livers of vaccinated ducks by immuno-staining for DHBcAg-positive hepatocytes but not in immuno-staining for DHBsAg-positive hepatocytes on day 4 p.c. (Table 5-2). In Chapter 3, the results show that immuno-staining for the detection of DHBsAg in EAA-fixed tissue and DHBcAg in formalin-fixed tissue have similar sensitivity, which detected 2.2% and 1.9% of positive staining hepatocytes respectively on liver tissue collected from a same duck and have similar distribution pattern within the liver lobules. The failure to detect DHBsAg- but DHBcAg-positive hepatocytes in the livers on day 4 p.c. could be due to the false manipulation that leads to low assay sensitivity, for example, the loss of the antigens in EAA fixative during the interval time from tissue removal to fixation.

On the other hand, following DHBV challenge, anti-DHBs antibodies were detected in 19 out of 21 ducks from Groups 5A-5D (except ducks 5A.6 and 5C.3) at day 4 p.c. or at later time points during the course of experiment. In contrast, anti-DHBs antibodies were not observed in all 5 out of 5 Group 5F ducks at any time during the course of experiment (Figure 5.4). It has been known that the resolution of DHBV infection is associated with the development of neutralising anti-DHBs antibodies (Jilbert and Kotlarski, 2000; Kuroki et al., 1995; Vickery et al., 1989). The ability of DNA vaccine expressing pre-S/S or S antigens to induce neutralising anti-DHBs antibodies has been demonstrated in many studies using the duck model (Miller et al., 2006b, 2008; Saade et al., 2008; Thermet et al., 2008; Noordeen Ph.D. Thesis, 2009; Saade et al., 2013). This suggested that the reduction of early virus replication in the livers in the ducks in Groups 5A-5D could be due to the binding of viruses by neutralising anti-DHBs antibodies induced by DNA vaccines expressing pre-S/S or S antigens. This facilitates the clearance of the virus from the blood and inhibits the interaction between the viruses and hepatocytes.
It is to note that patients with acute HBV infection are characterised by detectable HBV-specific CD4+ and CD8+ T responses with a Th1 profile of cytokine production (Bertoletti et al., 2006). The cytolytic or non-cytolytic mechanism of CTL induced after DNA vaccination could also prevent the cell-to-cell spread by killing the infected hepatocytes. Therefore, after DHBV challenge, DNA vaccination may play an important role in inducing specific T cell immune responses which resulted in the clearance of DHBV-infected hepatocytes in all 21 Group 5A-5D ducks. At this stage, we were unable to investigate the impact of DuCD40L expression construct on the induction of T cell responses as tools for analysis of CMI in the duck model are not available in our laboratory.

The surprising finding from this current study was only 3 out 5 Group 5E (DuCD40L control) ducks compared to five of five Group 5F (vector control) ducks developed persistent DHBV infection with >95% of DHBV-infected hepatocytes in the liver at day 21 p.c. Of the remaining two ducks in Group 5E, in duck 5E.2, anti-DHBs antibodies were constantly detectable in the serum with undetectable levels of DHBV DNA from day 7 p.c. until the end of the experiment, indicating the resolution of the DHBV infection. This observation was similar to other 14-day-old ducks challenged with 5x10^8 DHBV genomes that resolved DHBV infection (Foster et al., 2005; Miller et al., 2006b, 2008; Noordeen Thesis, 2009; Feng et al., 2010). In duck 5E.3, anti-DHBs antibodies were only transiently detected in the serum with 38.5% of DHBsAg-positive hepatocytes in the liver, reflecting unsuccessful attempts by the anti-DHBs antibodies to neutralise the infectivity of the virus or to form immune-complex with the virus to facilitate the removal of the virus from the circulation (Chang and Lewin, 2007). In addition, fluctuations in levels of DHBV DNA in the serum of ducks 5E.2 and 5E.3 could be due to the CMI against the infected hepatocytes attempting to control virus replication.
The findings in this current study were compatible with the results in Chapter 4 which showed only 7 out of the 11 ducks vaccinated with DuCD40L developed persistent DHBV infection following DHBV challenge. Since persistent DHBV infection was successfully established in all Group 5F (vector control) ducks, it is believed that the clearance/partial clearance of virus infection in Group 5E (DuCD40L control) ducks were not due to natural resolution of DHBV infection. While experimental evidence indicates that DuCD40L alone may enhance immunity of ducks against DHBV challenge, its capacity to enhance immune responses against DHBV infection in the liver is less clear.

Following virus challenge, it is proposed that CD40L could regulate the cellular and humoral responses in four ways: (1) CD40L induces the full activation of APC, prolongs the survival of activated APC and subsequently activates more naive CD4+ T lymphocytes that recognise MHC-presented antigens (Daoussis et al., 2004; Fraser et al., 2007) (2) CD40L induces APC to secrete pro-inflammatory and stimulatory cytokines such as TNF-α and interleukin (IL)-12. The cytokine TNF-α stimulates the activation of macrophages and IL-12 is required for the development of Th1 CD4+ T lymphocytes. In addition, IL-12 is also important in stimulating NK cells and T lymphocytes to secret anti-viral cytokines (e.g. IL-2 and IFN-γ) to control virus replication (Kornbluth, 2000) (3) CD40L activates (“license”) DC via CD40 to induce expression of co-stimulatory molecules (e.g. B7) that is necessary to prime CTL that already response to MHC-presented antigens, also known as cross-priming of CTL (Bennett et al., 1998; Smith et al., 2004). The virus infection is then controlled by antiviral CTL responses which include cytolytic and non-cytolytic mechanisms as previously described in Section 1.4.1.1.2. (4) CD40L activates B lymphocytes and induces B lymphocytes, differentiation, proliferation and Ig isotype switching (Garside et al., 1998; Aruffo et al., 1993). All these suggest that CD40L is a potential genetic adjuvant that can increase the cellular and humoral immune responses against virus infection.
However, the mechanism responsible for the protection seen in this study remains to be elucidated. This is because the assays to study the CMI in ducks were not available in our laboratory. Therefore, in the future study, to monitor the immune responses following vaccination and virus challenge in ducks, immuno-staining of CD4+ and CD8+ T lymphocytes using anti-duck CD4 and CD8 antibodies (Kothlow et al., 2005) could be performed. Recently, quantitative reverse transcription PCR (qRT-PCR) assays and specific primers for detection of the mRNA expression of duck cytokines IL-2, TNF-α and IFN-γ have been developed in our laboratory (Reaiche, Ph.D. Thesis, 2008). The qRT-PCR assays could be used to detect the mRNA expression of duck cytokines IL-2, TNF-α and IFN-γ to have a better understanding of the role of these cytokines against virus infection. Also, if possible, ELISPot assay could be developed to determine the frequency of Th1 and Th2 cytokines secreted by T lymphocytes, to gain more insight about the immunological events following vaccination and virus challenge.

Overall, our findings in Chapters 4 and 5 suggest that DuCD40L has the potential to be an effective genetic adjuvant in enhancement of the specific immune responses to DHBV antigens delivered by DHBV DNA vaccines. In this regard, a long-term larger study is required to confirm these findings and study on the effect of DHBV DNA vaccine combined with DuCD40L expression construct on DHBV total and cccDNA will also be required.
Chapter 6: The development of persistent DHBV infection can be prevented using antiviral ETV therapy combined with “prime-boost” vaccination with either DHBV surface or core antigens.

6.1 Introduction and aims

The currently available protective HBV vaccines provide no therapeutic benefit to patients with CHB (Fabriziet al., 2004). In patients who resolve acute HBV infection, vigorous, strong and multi-specific CD4+ and CD8+ T-cell responses toward to HBV proteins (e.g. HBsAg and HBcAg) can be detected, along with secretion of antiviral cytokines (e.g. IFN-α) and the production of neutralising anti-HBs antibodies (Akbar et al., 1999; Kara et al., 2004; Bertoletti and Gehring, 2006; Chang and Lewin, 2007; Chisari et al., 2010; Michel et al., 2011). In contrast, patients suffering from CHB have undetectable, weak or functionally impaired HBV-specific T cell responses (Rehermann et al., 1996; Yim and Lok, 2006; Kwon and Lok, 2011). Based on these observations, current therapeutic vaccination strategies aim to stimulate or broaden the host HBV-specific immune response to help eliminate CHB.

Previous studies have indicated that in patients resolving their acute HBV infection, a reduction in viral load precedes the strong and robust T-cell responses (Manno et al., 2004; Kara et al., 2004; Kwon and Lok, 2011). Therefore, one rational vaccination strategy to eliminate CHB would be to first to reduce viral load by a potent antiviral treatment and then to stimulate the specific T-cell responses by therapeutic vaccination.

Here, we proposed to use ETV antiviral treatment in combination with a heterologous “prime-boost” vaccination strategy that utilises sequential immunisation with DNA vaccines to “prime” followed by rFPV-vaccines to “boost” the specific immune responses. In this Chapter, the feasibility of this novel vaccination strategy was tested using DHBV model.
ETV has been approved in Australia as one of the antiviral drugs for treatment of CHB (Hepatitis Australia, 2010). At present, TFV and ETV are the only 2 NAs recommended by international guidelines as the first-line treatment for CHB (Carosi et al., 2011; European Association for the Study of the Liver, 2012). It has been shown that the active triphosphate form of ETV has a high affinity for the hepadnaviral polymerase and acts as a potent competitive inhibitor of natural substrate (dGTP) which results in chain termination (Xu and Chen, 2006; Ferir et al., 2008). ETV treatment has proved effective in CHB patients also in suppressing HBV DNA replication, improving liver histology with reduced ALT levels and HBeAg loss (Chang et al., 2006; Lai et al., 2006; Suzuki et al., 2008; Chen et al., 2011; Spaziante et al., 2014). In addition, patients treated with ETV rarely develop antiviral drug resistant mutants (Min and Dienstag, 2007; Hepatitis Australia, 2010). A recent study from Tenny et al., 2009 showed that after 6 years of ETV treatment, the cumulative rate of ETV-resistant mutants remains low at 1.2%. The relatively high genetic barrier, which requires at least 3 sites of genetic mutation to confer resistance to ETV has attributed to the low rate of resistance (Halegoua-De Marzio and Hann, 2014). Nonetheless, elimination of cccDNA is usually not achieved with the current antiviral approaches (Ferir et al., 2008). NA treatment can only suppress HBV replication but not completely eliminate the virus. Antiviral studies also concluded that the restoration of T-cell activities induced by NAs is transient and not sustainable, and rebound of HBV infection was usually observed in a majority of patients after the withdrawal of NA treatment (Bertoletti and Naoumov, 2003; Boni et al., 2001, 2003; Hoofnagle, 2006).

The DHBV model has been used extensively in preclinical studies of antiviral drugs designed for the treatment of CHB (Foster et al., 2003; Zoulim et al., 2008) and much of what we know about hepadnavirus pathogenesis and replication was identified from studies of the DHBV model (Mason et al., 1980; Newbold et al., 1995; Jilbert et al., 1996; Miller et al., 2004; Schultz et al., 2004; Le Mire et al., 2005; Reaiche et al., 2010; Reaiche-Miller et al., 2013;
Saade et al., 2013). In recent work using the DHBV model, ETV has been extensively used alone (Foster et al., 2005) or in combination with different forms of DNA and recombinant vaccines (Miller et al., 2008; Feng et al., 2010) for testing novel antiviral and vaccination approaches for DHBV infection. Previously, we reported that 14-day-old ducks treated with ETV from the time of DHBV infection until 14 or 49 days p.i. did not prevent initial DHBV infection in the liver (ETV is unable to completely block conversion of rcDNA to cccDNA during initiation of infection) but significantly reduced the spread of infection with at least 1000-fold fewer infected hepatocytes by day 7 p.i., which allowed ~50% of the ducks to fully recover from DHBV infection (Foster et al., 2005). In contrast, all non-ETV treated ducks developed persistent DHBV infection. The study suggested that ETV treatment restricted initial infection allowing the ETV-treated ducks to elicit effective antiviral immune responses to control DHBV infection. From this study, we can assume that in young ducks (e.g. 14-day-old ducks) the infection outcome is responsive to immune therapy, thus providing a sensitive model to develop and evaluate vaccination protocols.

Heterologous “prime-boost” vaccination strategies have been shown to be capable of inducing broad HBV-specific immune responses, particularly humoral and CMI responses in animal models and healthy human volunteers (Cavenaugh et al., 2010; Kosinska et al., 2012; Kosinska et al., 2013). A recent study in a chimpanzee with persistent HBV infection with a low virus load has shown that Lamivudine treatment in combination with “prime-boost” vaccination using DNA vaccines and canarypox viruses that express HBsAg, resulted in a reduction in virus load initially and finally led to the resolution of HBV infection (Shata et al., 2006).

In our most recent studies, therapeutic protocols for persistent DHBV infection that included ETV, DHBV DNA vaccines and rFPV strains expressing the DHBV surface and core antigens were performed in the DHBV model (Miller et al., 2008; Feng et al., 2010). As
described in Section 1.8.6, the rFPV-DHBV strains were derived from the FPV vaccine strain, FPV-M3 (Boyle et al., 1997). The rFPV-DHBV strains expressing DHBV core and pre-surface antigens (rFPV-DHBc and rFPV-DHBpre-S/S) can enter but do not replicate in mammalian cells, leading to expression of the cloned antigens and development of specific immune responses (Miller et al., 2008).

In Miller et al., 2008, 14-day-old ducks were infected with DHBV and were treated with ETV on day 1 p.i. for 14 days, and at the same time “primed” with DHBV DNA vaccines encoding DHBV surface and core antigens, followed by “boosting” with both rFPV-DHBc and rFPV-DHBpre-S/S on day 7 p.i. The results showed that ETV treatment and “prime-boost” vaccination prevented the development of persistent DHBV infection in 100% of the treated ducks (10/10 ducks). This finding indicates that ETV treatment in combination with heterologous “prime-boost” vaccination can enhance and provide additional immune responses against DHBV infection, which eventually prevented the development of persistent DHBV infection. In contrast only 50% of ducks treated with ETV alone and inoculated with an equal dose of DHBV successfully cleared their DHBV infection.

In a related study, 14-day-old ducks infected with 5x10^8 DHBV genomes and treated with ETV combined with either the DHBV DNA vaccines encoding surface and core antigens on day 0 p.i. or the rFPV-DHBc and rFPV-DHBpre-S/S vaccines on day 7 p.i. had no detectable DHBV-infected hepatocytes by day 14 p.i. and were protected from the development of persistent DHBV infection (Feng et al., 2010). In contrast, untreated control ducks infected with an equal dose of DHBV all developed persistent DHBV infection. The study also showed that the combination of DHBV DNA vaccine “prime” and rFPV-DHBV vaccine “boost” stimulated higher levels of production of protective anti-DHBs antibodies than the DHBV DNA vaccines or rFPV-DHBV vaccines alone.
As mentioned before, cccDNA represents the intracellular HBV template (Newbold et al., 1995). It is estimated that ~1-50 copies of cccDNA present in individual infected hepatocytes (Jilbert et al., 1992; Kajino et al, 1994; Zhang et al., 2003). Several studies in animal models and in patients with CHB have clearly shown that nuclear cccDNA accumulated in infected hepatocytes serves as the template for viral transcription that maintains persistent infection (Mason et al., 1994; Michalak et al., 1994; Yotsuyanagi et al., 1998; Le Mire et al., 2005; Lai et al., 2010). Studies in DHBV infected ducks revealed that ~80% of liver residual DHBV DNA is in the cccDNA form, indicating the crucial role of cccDNA in the persistence and reactivation of the virus (Le Mire et al., 2005; Reaiche et al., 2010; Reaiche-Miller et al., 2013).

In patients with resolved HBV infection, traces of residual HBV DNA can be detected in patient serum and liver years after resolution of HBV infection (Michalak et al., 1994; Yotsuyanagi et al., 1998; Raimondo et al., 2010). In addition, HBV reactivation has been observed following treatment with immunosuppressive drugs (Hu, 2002; Mulrooney-Cousins and Michalak, 2007). The reactivation of HBV infection could be related to the presence of residual HBV cccDNA, which is normally controlled by immune responses but can be reactivated during immuno-suppressive therapy in the patients with resolved HBV infection. Therefore, elimination of the cccDNA reservoir from infected hepatocytes is thought to be important in the resolution of CHB.

During resolution of HBV infection, CTL play a major role in reducing the levels of circulating virus and clearance of HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms. Analysis of WHV and DHBV infections in woodchucks and ducks suggested that the resolution of acute infection involved cytolytic hepatocyte killing rather than non-cytolytic process (Guo et al., 2000; Jilbert et al., 1992; Kajino et al., 1994; Summers et al., 2003). In contrast, it has been proposed that cccDNA and all other viral DNA and RNA
could be eliminated through the non-cytolytic mechanism involves a wide range of anti-viral cytokines including IFN-α, IFN-γ and TNF-α without hepatocyte death (Guidotti and Chisari, 2001; Guidotti, 2002; Thimme et al., 2003). In the work described in Reaiche et al., 2010, the data suggest that that residual cccDNA is highly stable with a turnover rate similar to normal uninfected hepatocytes. A more recent study suggests that cccDNA survives hepatocyte mitosis in the growing liver (Reaiche-Miller et al., 2013). Taken together, it is believed that complete resolution of HBV infection is only possible when cccDNA is permanently eliminated by a potent CMI response that targets infected hepatocytes. Destruction of hepatocytes by HBV-specific CTL might be the major route for clearance of virus and liver cccDNA, to prevent the rebound of virus replication following the cessation of therapy.

As mentioned previously, neutralising epitopes within the DHBV pre-S/S and S proteins have been identified; three epitopes recognised by neutralising antibodies were found in pre-S/S domain and only one was found in the S domain (Cheung et al., 1989). The effectiveness of DNA immunisation to DHBV pre-S/S and S antigens has also been shown to induce strong, specific and highly neutralising anti-DHBs antibody responses (Triyatni et al., 1998; Rollier et al., 1999; Miller et al., 2006b; Saadeet al., 2008). In contrast to DHBV pre-S/S and S proteins, DNA vaccination of ducks with plasmid expressing DHBV core antigen induced non-neutralising antibody responses, which confer no protection to DHBV infection (Vickery et al., 1989; Jilbert and Kotlarski, 2000; Miller et al., 2008; Feng et al., 2010). In DHBV core protein, six antigenic regions (AR1 to AR6) have been identified using peptide scanning (Thermet et al., 2004). Recent studies in our laboratory showed that novel whole cell vaccines expressing DHBV core antigen induced antiviral immune responses that enabled rapid clearance of de novo infection (Miller et al., 2006a).

As the next step toward developing a potential therapeutic DHBV vaccine, in this Chapter, a post-exposure vaccination study was performed to determine if DHBV surface antigen which
generates neutralising anti-DHBs antibodies, or DHBV core antigen which generates non-neutralising anti-DHBc antibodies, could provide the essential epitopes in a DHBV DNA vaccine and rFPV-DHBV “prime-boost” protocol to prevent the development of persistent DHBV infection.

6.2 Experimental design

In this study, twenty-five DHBV negative ducks were divided into five Groups: Groups 6A – 6E (Table 6-1). At 14 days of age, ducks were inoculated i.v. with $5 \times 10^8$ DHBV genomes (Pool 8) as described in Section 2.5.1 and oral treatment with ETV (1.0 mg/kg/day for 14 days) as described in Section 2.5.2.3 was commenced in Groups 6A, 6B, 6C and 6D. Group 6E was treated with water. At the same time, the ducks were “primed” i.m. with 500 μg each construct: pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C (Group 6A), or pcDNA3-pre-S/S and pcDNA3-S (Group 6B), or pcDNA3-C alone (Group 6C), or the pcDNA3 vector only (Group 6D and 6E). At day 7 p.i., ducks were “boosted” with rFPV-DHBpre-S/S (Group 6A and 6B), rFPV-DHBc (Group 6A and 6C), or FPV-M3 the parent FPV vaccine strain (Group 6D and 6E) at a dose of $5 \times 10^7$ p.f.u. for each duck as described in Section 2.5.2.2. The outline of this experiment is shown in Figure 6.1.

All ducks were bled weekly and serum samples were analysed for: (i) levels of DHBsAg in the serum by rapid qualitative ELISA as described in Section 2.6.1. (ii) levels of anti-DHBs antibodies in the serum by quantitative ELISA as described in Section 2.6.2. (iii) levels of anti-DHBc antibodies in the serum by quantitative ELISA as described in Section 2.6.3 and (iv) levels of serum DHBV DNA. Serum samples were extracted using the ChargeSwitchgDNA 1 mL serum kit as described in Section 2.8.1 and served as templates for
serum qPCR assay using primer set 423 – 567c (Table 2-6) as described in Sections 2.8.3 and 2.8.4.

Liver samples obtained from biopsies (day 14 p.i.) and autopsies (day 63 p.i.) were used for: (i) immuno-staining of DHBsAg using EAA-fixed duck liver tissues using monoclonal anti-DHBV pre-S/S antibodies, 1H.1 (Pugh et al., 1995) to determine the percentage of DHBsAg-positive hepatocytes as described in Section 2.7.1.; (ii) immuno-staining of DHBcAg using formalin-fixed duck liver tissues treated with an antigen retrieval protocol followed by immuno-staining with polyclonal anti-DHBc antibodies, CQT-2, as described in Section 2.7.2. (iii) qPCR analysis of total DHBV and cccDNA DNA after DNA extraction using a DNeasy® Blood and tissue kit as described in Section 2.8.2 and quantitative qPCR using the primer sets 423-576c and CC2–MG1 respectively (Table 2-6) as described in Sections 2.8.3 and 2.8.5.

6.3 Results

6.3.1 Group A: DHBV infection outcomes in ducks that received ETV treatment in combination with “prime-boost” vaccines that expressed DHBV surface and core antigens.

Analysis of the liver tissue collected at day 14 and 63 p.i. indicated that the ETV treated and “prime-boost” vaccinated ducks in Groups 6A (ETV + surface and core) had no detectable or <0.001% DHBsAg-positive (and DHBcAg-positive) hepatocytes in the liver (Table 6-2), suggesting that all 5/5 Group 6A ducks had cleared their DHBV infection.
Table 6-1. The summary of experimental groups in Chapter 6

<table>
<thead>
<tr>
<th>Group</th>
<th>DHBV infection</th>
<th>ETV treatment</th>
<th>Water treatment</th>
<th>DHBV surface antigen</th>
<th>DHBV core antigen</th>
<th>PcDNA3 vector</th>
<th>FPV-M3 vector</th>
<th>Number of ducks</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>6B</td>
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</tr>
<tr>
<td>6D</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>6E</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>

aEach group was defined by the “prime-boost” vaccination given to the ducks with or without ETV treatment.

bAt 14 days of age, all twenty-five ducks were inoculated i.v. with 5x10^8 DHBV genomes, a dose that is 500 times higher than that previously shown to cause persistent DHBV infection in 14-day-old ducks (Jilbert et al., 1998; Foster et al., 2005; Miller et al., 2006b).

cETV treatment (1.0 mg/kg/day, oral) was commenced at day 0 p.i. until day 14 p.i. for 14 days after DHBV inoculation.

dWater treatment was commenced at day 0 p.i. until day 14 p.i. for 14 days after DHBV inoculation.

eDucks were “primed” i.m. with DNA vaccines that expressed DHBV surface (pre-S/S and S) antigens at 14 days of age and were “boosted” i.m. with rFPV vaccines that also expressed DHBV pre-S/S antigen at 21 days of age.

fDucks were “primed” i.m. with DNA vaccines that expressed DHBc antigen at 14 days of age and were “boosted” i.m. with rFPV vaccines that expressed DHBc antigens at 21 days of age.

gDucks were “primed” i.m. with DNA vector at 14 days of age and were “boosted” i.m. with FPV-M3 at 21 days of age.
Figure 6.1. The schedule of virus inoculation, oral ETV treatment, “prime-boost” vaccination, and liver biopsies and autopsy in 14-day-old ducks

Outline of the experiment described in Chapter 6. Twenty-five, DHBV negative ducks were divided into 5 Groups, Groups 6A – 6E. In this study, four plasmid DNA constructs were used as previously described in Chapters 4 and 5: pcDNA3-pre-S/S, pcDNA3-S, pcDNA3-C and pcDNA3. At 14 days of age, ducks were inoculated i.v. with $5 \times 10^8$ DHBV genomes (Pool 8) as described in Section 2.5.1 and oral treatment with ETV (1.0 mg/kg/day for 14 days) as described in Section 2.5.2.3 was commenced in Groups 6A, 6B, 6C and 6D. Group 6E was treated with water. At the same time, the ducks were “primed” i.m. with 500 μg of each DNA vaccine: pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C (Group 6A), or pcDNA3-pre-S/S and pcDNA3-S (Group 6B), or pcDNA3-C alone (Group 6C), or the pcDNA3 vector only (Group 6D and 6E). At day 7 p.i., ducks were “boosted” i.m. with rFPV-DHBc (Group 6A and 6C), rFPV-DHBpre-S/S (Group 6A and 6B) or the parent FPV vaccine strain, FPV-M3 (Group 6D and 6E) at a dose of $5 \times 10^7$ p.f.u. for each duck as described in Section 2.5.2.2.
Legend for Table 6-2.

Duck liver tissues were fixed, embedded and sectioned as described in Section 2.5.7. The outcomes of DHBV infection in different treatment groups were determined by measuring the percentage of DHBsAg- and DHBcAg-positive hepatocytes on days 14 and 63 p.i.

\(^a\) Each group was defined by the “prime-boost” vaccination given to the ducks with or without ETV treatment.

\(^b\) Immuno-staining of DHBsAg-positive hepatocytes were performed in EAA-fixed tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in Section 2.7.1.

\(^c\) Immuno-staining of DHBcAg-positive hepatocytes were performed in antigen retrieval treated formalin-fixed tissues with anti-DHBc polyclonal antibodies (CQT-2) as described in Section 2.7.2.

\(^d\) Duck liver tissues were collected at biopsy on day 14 p.i. and at autopsy on day 63 p.i. as described in Section 2.5.5 and 2.5.6 respectively.

\(^e\) The minimum sensitivity of detection of DHBsAg-positive hepatocytes (0.001%) was based on counting 100,000 hepatocytes in sections of liver tissue collected at biopsy and at autopsy.

\(^f\) Liver tissue sections where all observed hepatocytes were DHBsAg/DHBcAg positive were recorded as having >95% of DHBsAg-positive hepatocytes.
Table 6-2. The percentage of DHBsAg- and DHBcAg-positive hepatocytes on day 14 and 63 p.i.

<table>
<thead>
<tr>
<th>Groups&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Duck No</th>
<th>Duck</th>
<th>% DHBsAg-positive hepatocytes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% DHBcAg-positive hepatocytes&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 p.i.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>63 p.i.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>6A</td>
<td>205/206</td>
<td>6A.1</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>207/208</td>
<td>6A.2</td>
<td>&lt;0.001&lt;sup*e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>209/210</td>
<td>6A.3</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>211/212</td>
<td>6A.4</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>213/214</td>
<td>6A.5</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>6B</td>
<td>215/216</td>
<td>6B.1</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>217/218</td>
<td>6B.2</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>220/221</td>
<td>6B.3</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>222/223</td>
<td>6B.4</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>225/227</td>
<td>6B.5</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>6C</td>
<td>228/229</td>
<td>6C.1</td>
<td>0.01</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>230/231</td>
<td>6C.2</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>232/233</td>
<td>6C.3</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>234/235</td>
<td>6C.4</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>236/237</td>
<td>6C.5</td>
<td>0.006</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>6D</td>
<td>238/239</td>
<td>6D.1</td>
<td>0.35</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>240/241</td>
<td>6D.2</td>
<td>0.02</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>242/243</td>
<td>6D.3</td>
<td>0.45</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>244/245</td>
<td>6D.4</td>
<td>0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>246/247</td>
<td>6D.5</td>
<td>0.04</td>
<td>2.4</td>
</tr>
<tr>
<td>6E</td>
<td>248/249</td>
<td>6E.1</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>250/251</td>
<td>6E.2</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>252/253</td>
<td>6E.3</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>254/255</td>
<td>6E.4</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>256/257</td>
<td>6E.5</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt;95&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 6-3. Statistical analysis of the differences of the percentage of DHBsAg-positive hepatocytes on day 14 and 63 p.i.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Day 14 p.i.</th>
<th>Day 63 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean differences (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Group 6A vs. Group 6B</td>
<td>0 (-0.117, 0.117)</td>
<td>0 (-24.867, 24.867)</td>
</tr>
<tr>
<td></td>
<td>p = 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p = 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6A vs. Group 6C</td>
<td>-0.003 (-0.12, 0.114)</td>
<td>0 (-24.867, 24.867)</td>
</tr>
<tr>
<td></td>
<td>p = 0.9946&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p = 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6B vs. Group 6C</td>
<td>-0.003 (-0.120, 0.114)</td>
<td>0 (-24.867, 24.867)</td>
</tr>
<tr>
<td></td>
<td>p = 0.9606&lt;sup&gt;b&lt;/sup&gt;</td>
<td>p = 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6A vs. Group 6D</td>
<td>-19.623 (-44.49, 5.244)</td>
<td>-0.187 (-0.304, -0.07)</td>
</tr>
<tr>
<td></td>
<td>p = 0.1154&lt;sup&gt;c&lt;/sup&gt;</td>
<td>p = 0.0033&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6B vs. Group 6D</td>
<td>-19.623 (-44.49, 5.244)</td>
<td>-0.187 (-0.304, -0.07)</td>
</tr>
<tr>
<td></td>
<td>p = 0.1154&lt;sup&gt;c&lt;/sup&gt;</td>
<td>p = 0.0033&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6C vs. Group 6D</td>
<td>-19.62 (-44.49, 5.244)</td>
<td>-0.184 (-0.301, -0.067)</td>
</tr>
<tr>
<td></td>
<td>p = 0.1154&lt;sup&gt;c&lt;/sup&gt;</td>
<td>p = 0.0037&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6D vs. Group 6E</td>
<td>-94.81 (-94.929, -94.5695)</td>
<td>-75.29 (-100.243, -50.51)</td>
</tr>
<tr>
<td></td>
<td>p = &lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
<td>p = &lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Statistical analysis was performed as reported in Section 2.9.

<sup>b</sup> The percentage of DHBsAg-positive hepatocytes was not significantly different in ducks in Groups 6A vs. 6B (p = 1), Groups 6A vs. 6C (p = 9946; p = 1), and Groups 6B vs. 6C (p = 0.9606; p = 1), at day 14 and 63 p.i. This indicated that DHBV surface and core antigen or a combination of two as measured in our studies is equally effective as components of our post-exposure “prime-boost” protocol.

<sup>c</sup> Differences in the average percentage of DHBsAg-positive hepatocytes between the ducks in Group 6A vs. 6D, Group 6B vs. 6D and Group 6C vs. 6D was not significantly different (p = 0.1154) at day 14 p.i. due to the effect of ETV treatment on reducing the spread of DHBV infection, but was statistically significant (p = 0.0033; p = 0.0037) at day 63 p.i. reflecting the effect of “prime-boost” vaccination on altering the outcome of the DHBV infection.

<sup>d</sup> Differences in the average percentage of DHBsAg-positive heptocytes between the ducks in Group 6D vs. 6E were highly statistically significant at all time-points (p < 0.0001).
Table 6-4. Statistical analysis of the differences of the percentage of DHBcAg-positive hepatocytes on day 14 and 63 p.i.

<table>
<thead>
<tr>
<th>Comparison&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 14 p.i.</th>
<th>Day 63 p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean differences (95% CI)</td>
<td><em>p</em> value</td>
</tr>
<tr>
<td>Group 6A vs. Group 6B</td>
<td>0 (-0.184, 0.184)</td>
<td>0 (-24.836, 24.836)</td>
</tr>
<tr>
<td></td>
<td><em>p</em> = 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>p</em> = 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6A vs. Group 6C</td>
<td>-0.0006 (-0.19, 0.18)</td>
<td>0 (-24.836, 24.836)</td>
</tr>
<tr>
<td></td>
<td><em>p</em> = 0.9946&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>p</em> = 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6B vs. Group 6C</td>
<td>-0.001 (-0.185, 0.184)</td>
<td>0 (-24.836, 24.836)</td>
</tr>
<tr>
<td></td>
<td><em>p</em> = 0.9946&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>p</em> = 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6A vs. Group 6D</td>
<td>-19.713 (-44.549, 5.123)</td>
<td>-0.223 (-0.407, -0.039)</td>
</tr>
<tr>
<td></td>
<td><em>p</em> = 0.1134&lt;sup&gt;c&lt;/sup&gt;</td>
<td><em>p</em> = 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6B vs. Group 6D</td>
<td>-19.713 (-44.549, 5.123)</td>
<td>-0.223 (-0.407, -0.039)</td>
</tr>
<tr>
<td></td>
<td><em>p</em> = 0.1134&lt;sup&gt;c&lt;/sup&gt;</td>
<td><em>p</em> = 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6C vs. Group 6D</td>
<td>-19.713 (-44.549, 5.123)</td>
<td>-0.223 (-0.407, -0.039)</td>
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<tr>
<td></td>
<td><em>p</em> = 0.1134&lt;sup&gt;c&lt;/sup&gt;</td>
<td><em>p</em> = 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 6D vs. Group 6E</td>
<td>-94.775 (-94.959, -94.592)</td>
<td>-75.29 (-100.122, -50.45)</td>
</tr>
<tr>
<td></td>
<td><em>p</em> = &lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
<td><em>p</em> = &lt;0.0001&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Statistical analysis was performed as reported in Section 2.9.

<sup>b</sup>The percentage of DHBcAg-positive hepatocytes was not significantly different in ducks in Groups 6A vs. 6B (*p* = 1). Groups 6A vs. 6C (*p* = 0.9946; *p* = 1) and Groups 6B vs. 6C (*p* = 0.9946; *p* = 1) at day 14 and 63 p.i. This indicated that DHBV surface and core antigen or a combination of two as measured in our studies was equally effective as components of our post-exposure “prime-boost” protocol.

<sup>c</sup>Differences in the average percentage of DHBcAg-positive hepatocytes between the ducks in Group 6A vs. 6D, Group 6B vs. 6D and Group 6C vs. 6D were not significantly different (*p* = 0.1134), at day 14 p.i. due to the effect of ETV treatment on reducing the spread of DHBV infection, but was statistically significant (*p* = 0.02) at day 63 p.i. reflecting the effect of “prime-boost” vaccination on altering the outcome of the DHBV infection.

<sup>d</sup>Differences in the average percentage of DHBcAg-positive hepatocytes between the ducks in Group 6D vs. 6E were highly statistically significant (*p* < 0.0001) at all time-points.
Figure 6.2. DHBsAg levels in the sera measured by rapid qualitative ELISA as described in Section 2.6.1. for each duck from before inoculation (pre-bleed) until day 77 of age (63 p.i.).

Twenty-five 14-day-old ducks were inoculated i.v. with 5x10^8 DHBV genomes (Pool 8) and were vaccinated as described in the legend to Figure 6.1.

Panel A: Group 6A ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C and rFPV strains, rFPV-DHBpre-S/S and rFPV-DHBc.

Panel B: Group 6B ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S and pcDNA3-S and rFPV strain, rFPV-DHBpre-S/S.

Panel C: Group 6C ducks received ETV, DHBV DNA vaccine pcDNA3-C, and rFPV strain, rFPV-DHBc.

Panel D: Group 6D ducks received ETV and control vectors (pcDNA3 and FPV-M3).

Panel E: Group 6E ducks received water and control vectors (pcDNA3 and FPV-M3).

The levels of DHBsAg present in the serum were calculated based on standard curves generated using the Pool 8 serum containing 50 µg/mL of DHBsAg (Jilbert et al., 1996). The background level of the DHBsAg ELISA was determined by the mean O.D. readings of NDS plus two standard deviations. The background reading (0.48 µg/mL of DHBsAg) is shown as dotted line in Figure 6.2 and used as the limit of detection for all these assays.
DHBV DNA vaccines
rFPV-DHBV vaccines
pcDNA3 control vector
FPV-M3
DHBV challenge
ETV
water

Age (days)
DHBV challenge
DHBV DNA vaccines
rFPV-DHBV vaccines
pcDNA3 control vector
FPV-M3
ETV
water
In agreement with the liver analysis, analysis of the serum samples indicated that the Group 6A ducks had developed low levels of serum DHBsAg, which duck 6A.4 had the highest DHBsAg (0.77 µg/mL) detected at day 56 (42 p.i.) (Figure 6.2A), indicating that levels of virus replication were low in the liver of the Group 6A ducks. Following DHBV infection and vaccination, two ducks from Group 6A, ducks 6A.1 and 6A.5, had developed high titres of anti-DHBs antibodies more rapidly than those ETV-treated ducks vaccinated without surface antigens in Group 6C (ETV + core) by day 28 (14 p.i.) (Figure 6.3A). Similarly, Group 6A ducks had also developed high titres of anti-DHBc antibodies more rapidly than those ETV-treated ducks vaccinated without pcDNA3-C in Group 6B (ETV + surface) (Figure 6.4A). The rapid humoral immune response detected in the Group 6A ducks could be solely due to the effect of DHBV DNA vaccination since virus replication was still inhibited by ETV on day 28 (14 p.i.), showing the ability of DHBV DNA vaccines to enhance the production of anti-DHBs and anti-DHBc antibodies.

As mentioned earlier, liver cccDNA is important for both the persistence and reactivation of the virus (Le Mire et al., 2005; Reaiche et al., 2010; Reaiche-Miller et al., 2013). Therefore, biopsy (day 14 p.i.) and autopsy (day 63 p.i.) liver tissue from the Group 6A ducks was extracted and subjected to qPCR analysis for DHBV total and cccDNA. Since DHBsAg-positive and DHBcAg-positive hepatocytes were no longer detected in the liver (<0.001%), any DHBV DNA (DHBV total and cccDNA) detected on day 14 and 63 p.i. is considered to be residual DNA.

The results showed that Group 6A ducks had lower levels of total DHBV DNA (Range: 0.0002-0.002 and 0.0011-0.086 copies/cell respectively) (Figure 6.5A) than Group 6E ducks (water + control vector) on day 14 and 63 p.i. (Range: 22.6–103.6 and 58.6–108.6 copies/cell respectively) (Figure 6.5). Again, Group 6A ducks had lower levels of levels of cccDNA (Range: 0.0001-0.0003 and 0.0001-0.0003 copies/cell respectively) (Figure 6.5) than the
Group 6E ducks (Range: 11.40-18.1 and 37.8-69.81 copies/cell respectively) on day 14 and 63 p.i. (Figure 6.5). The decrease in the levels of liver total and cccDNA in Group 6A compared to Group 6E indicated that ETV treatment in combination with “prime-boost” vaccination had successfully induced immune responses to prevent the development of persistent DHBV infection. In patients with resolved HBV infection, despite the presence of neutralising anti-HBs antibodies, traces of residual HBV DNA can still be detected in the serum and liver years after resolution of HBV infection (Michalak et al., 1994; Yotsuyanagi et al., 1998; Raimondo et al., 2010). Therefore, the presence of low levels of residual DHBV DNA was not unexpected in Group 6A ducks that had resolved from DHBV infection.

In Group 6A, all 5/5 ducks had low levels of virus replication, cleared infected hepatocytes from the liver and did not develop persistent DHBV infection. Since only two ducks from Group 6A, ducks 6A.1 and 6A.5, had developed high titres of anti-DHBs antibodies (Figure 6.3), this suggested that other than humoral immune responses, specific CMI responses against DHBV surface and core antigens might also play a key role in DHBV clearance. However, we were unable to investigate the specific CMI responses in this study due to the limitation on duck specific reagents.

6.3.2 Group 6B: DHBV infection outcomes in ducks that received ETV treatment in combination of “prime-boost” vaccines that expressed DHBV surface antigens.

Analysis of the liver tissue collected at day 14 and 63 p.i. showed that all 5/5 ETV treated and “prime-boost” vaccinated ducks in Groups 6B (ETV + surface alone) had no detectable or <0.001% DHBsAg-positive (and DHBcAg-positive) hepatocytes in the liver (Table 6-2). In addition, serum DHBsAg detected by quantitative ELISA (Figure 6.2A-B) and the mean levels of liver DHBV total and cccDNA (Figure 6.6) were similar between Groups 6A and
Figure 6.3 Anti-DHBs antibodies levels in the sera measured by quantitative ELISA as described in Section 2.6.2. for each duck from before inoculation (pre-bleed) until day 77 on age (63 p.i.).

Twenty-five 14-day-old ducks were inoculated i.v. with $5 \times 10^8$ DHBV genomes (Pool 8) and were vaccinated as described in the legend to Figure 6.1.

Panel A: Group 6A ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C and rFPV strains, rFPV-DHBpre-S/S and rFPV-DHBc.

Panel B: Group 6B ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S and pcDNA3-S and rFPV strain, rFPV-DHBpre-S/S.

Panel C: Group 6C ducks received ETV, DHBV DNA vaccine pcDNA3-C, and rFPV strain, rFPV-DHBc.

Panel D: Group 6D ducks received ETV and control vectors (pcDNA3 and FPV-M3).

Panel E: Group 6E ducks received water and control vectors (pcDNA3 and FPV-M3).

Titres of anti-DHBs antibodies are expressed as the reciprocal of the serum dilution required to achieve an O.D. of 0.326 at 490 nm. The negative cut-off for anti-DHBs-positive samples was determined by the mean O.D. readings of NDS at a dilution of 1/100 plus two standard deviations. As the minimum serum dilution was 1/100, the minimum titre is 100 (shown as the bottom line in Figure 6.3).
DHBV DNA vaccines
rFPV-DHBV vaccines
pcDNA3 control vector
FPV-M3
DHBV challenge
ETV
water

Age (days)

Anti-DHBs antibodies

DHBV challenge
DHBV DNA vaccines
rFPV-DHBV vaccines
pcDNA3 control vector
FPV-M3
ETV
water
Figure 6.4. The anti-DHBc antibodies levels in the sera measured by quantitative ELISA as described in Section 2.6.3. for each duck from before inoculation (pre-bleed) until day 77 (63 p.i.).

Twenty-five 14-day-old ducks were inoculated i.v. with $5 \times 10^8$ DHBV genomes (Pool 8) and were vaccinated as described in the legend to Figure 6.1.

Panel A: Group 6A ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C and rFPV strains, rFPV-DHBpre-S/S and rFPV-DHBc.

Panel B: Group 6B ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S and pcDNA3-S and rFPV strain, rFPV-DHBpre-S/S.

Panel C: Group 6C ducks received ETV, DHBV DNA vaccine pcDNA3-C, and rFPV strain, rFPV-DHBc.

Panel D: Group 6D ducks received ETV and control vectors (pcDNA3 and FPV-M3).

Panel E: Group 6E ducks received water and control vectors (pcDNA3 and FPV-M3).

Titres of anti-DHBc antibodies are expressed as the reciprocal of the serum dilution required to achieve an O.D. of 0.250 at 490 nm. The negative cut-off for anti-DHBc-positive samples was determined by the mean O.D. readings of NDS at a dilution of 1 in 1000 plus two standard deviations. As the minimum serum dilution was 1/1000, the minimum titre is 100 (shown as the bottom line in Figure 6.4).
DHBV DNA vaccines
rFPV-DHBV vaccines
pcDNA3 control vector
FPV-M3
DHBV challenge
ETV
water

1.E+03
1.E+04
1.E+05
0 10 20 30 40 50 60 70 80

6A.1 6A.2 6A.3 6A.4 6A.5
1.E+03
1.E+04
1.E+05
0 10 20 30 40 50 60 70 80

6B.1 6B.2 6B.3 6B.4 6B.5
1.E+03
1.E+04
1.E+05
0 10 20 30 40 50 60 70 80

6C.1 6C.2 6C.3 6C.4 6C.5
1.E+03
1.E+04
1.E+05
0 10 20 30 40 50 60 70 80

6D.1 6D.2 6D.3 6D.4 6D.5
1.E+03
1.E+04
1.E+05
0 10 20 30 40 50 60 70 80

6E.1 6E.2 6E.3 6E.4 6E.5
1.E+03
1.E+04
1.E+05
0 10 20 30 40 50 60 70 80

Age (days)

DHBV challenge
DHBV DNA vaccines
rFPV-DHBV vaccines
pcDNA3 control vector
FPV-M3
ETV
water
Figure 6.5. The levels of DHBV total and cccDNA in the liver of ducks in Groups 6A, 6B, 6C, 6D and 6E as detected by qPCR reaction on days 14 and 63 p.i.

Twenty-five 14-day-old ducks were inoculated i.v. with 5x10^8 DHBV genomes (Pool 8) and were vaccinated as described in the legend to Figure 6.1.

Total cellular and viral DNA was extracted from biopsy and autopsy liver tissues as described in Section 2.8.2 and subjected to qPCR analysis for levels of DHBV total and cccDNA using primer sets 423-576c and CC2-MG1 (Table 2-6) as described in Section 2.8.5. The limit of detection of the assay shown as the dotted line in the Figure is determined by the limit of sensitivity of the qPCR which does not accurately detect less than 10 copies of total DHBV DNA per 53,571 cells, or 0.00019 copies of DHBV DNA per cell.

Panel A: Group 6A ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C and rFPV strains, rFPV-DHBpre-S/S and rFPV-DHBc.

Panel B: Group 6B ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S and pcDNA3-S and rFPV strain, rFPV-DHBpre-S/S.

Panel C: Group 6C ducks received ETV, DHBV DNA vaccine pcDNA3-C, and rFPV strain, rFPV-DHBc.

Panel D: Group 6D ducks received ETV and control vectors (pcDNA3 and FPV-M3).

Panel E: Group 6E ducks received water and control vectors (pcDNA3 and FPV-M3).
Table 6-5. Statistical analysis of the levels of a) DHBV total and b) cccDNA in the liver of ducks in Groups 6A, 6B, 6C, 6D and 6E on days 14 and 63 p.i.

a)

<table>
<thead>
<tr>
<th>Comparison a</th>
<th>Day 14 p.i.</th>
<th>Day 63 p.i.</th>
<th>Mean differences (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 6A vs. Group 6B</td>
<td>0 (-20.845, 20.845)</td>
<td>-0.0637 (-20.927, 20.8)</td>
<td>p = 1 b</td>
<td>p = 0.9950 b</td>
</tr>
<tr>
<td>p = 0.9999 b</td>
<td>p = 0.9998 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6A vs. Group 6C</td>
<td>-0.001 (-20.846, 20.844)</td>
<td>0.0002 (-20.866, 20.862)</td>
<td>p = 0.9999 b</td>
<td>p = 1 b</td>
</tr>
<tr>
<td>p = 0.9998 b</td>
<td>p = 0.9997 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6B vs. Group 6C</td>
<td>-0.001 (-20.846, 20.844)</td>
<td>0.062 (-20.802, 20.925)</td>
<td>p = 0.9999 b</td>
<td>p = 1 b</td>
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<tr>
<td>p = 0.9998 b</td>
<td>p = 0.997 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6A vs. Group 6D</td>
<td>-0.017 (-20.863, 20.828)</td>
<td>-15.869 (-36.732, 4.995)</td>
<td>p = 0.9998 c</td>
<td>p = 0.1283 c</td>
</tr>
<tr>
<td>p = 0.9986 c</td>
<td>p = 0.1298 c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6B vs. Group 6D</td>
<td>-0.017 (-20.862, 20.829)</td>
<td>-15.804 (-36.668, 5.058)</td>
<td>p = 0.9998 c</td>
<td>p = 0.1298 c</td>
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<tr>
<td>p = 0.9986 c</td>
<td>p = 0.1298 c</td>
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<tr>
<td>Group 6C vs. Group 6D</td>
<td>-0.016 (-20.861, 20.828)</td>
<td>-15.866 (-36.730, 4.997)</td>
<td>p = 0.9998 c</td>
<td>p = 0.1283 c</td>
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<tr>
<td>p = 0.9987 c</td>
<td>p = 0.1283 c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6D vs. Group 6E</td>
<td>-65.608 (-86.454, -44.763)</td>
<td>-60.786 (-81.65, -39.923)</td>
<td>p &lt; 0.0001 d</td>
<td>p &lt; 0.0001 d</td>
</tr>
<tr>
<td>p &lt; 0.0001 d</td>
<td>p &lt; 0.0001 d</td>
<td></td>
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<td></td>
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</tbody>
</table>

b)

<table>
<thead>
<tr>
<th>Comparison a</th>
<th>Day 14 p.i.</th>
<th>Day 63 p.i.</th>
<th>Mean differences (95% CI)</th>
<th>p value</th>
</tr>
</thead>
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<tr>
<td>Group 6A vs. Group 6B</td>
<td>0 (-1.723, 1.723)</td>
<td>0 (-10.651, 10.650)</td>
<td>p = 1 b</td>
<td>p = 0.9999 b</td>
</tr>
<tr>
<td>p = 1 b</td>
<td>p = 0.9999 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6A vs. Group 6C</td>
<td>0 (-1.723, 1.722)</td>
<td>0-0.001 (-10.651, 10.650)</td>
<td>p = 0.9999 b</td>
<td>p = 0.9999 b</td>
</tr>
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<td>p = 0.9999 b</td>
<td>p = 0.9999 b</td>
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<td></td>
<td></td>
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<tr>
<td>Group 6B vs. Group 6C</td>
<td>0 (-1.723, 1.722)</td>
<td>0 (-10.651, 10.650)</td>
<td>p = 0.9994 b</td>
<td>p = 1 b</td>
</tr>
<tr>
<td>p = 0.9994 b</td>
<td>p = 1 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6A vs. Group 6D</td>
<td>0 (-1.724, 1.722)</td>
<td>-5.450 (-16.102, 5.2)</td>
<td>p = 0.9997 c</td>
<td>p = 0.2984 c</td>
</tr>
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<td>p = 0.9991 c</td>
<td>p = 0.2984 c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6B vs. Group 6D</td>
<td>-0.001 (-1.724, 1.722)</td>
<td>-5.450 (-16.101, 5.2)</td>
<td>p = 0.9997 c</td>
<td>p = 0.2985 c</td>
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<td>p = 0.2985 c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6C vs. Group 6D</td>
<td>0 (-1.723, 1.723)</td>
<td>-5.450 (-16.101, 5.2)</td>
<td>p = 0.9997 c</td>
<td>p = 0.2985 c</td>
</tr>
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<td>p = 0.9997 c</td>
<td>p = 0.2985 c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 6D vs. Group 6E</td>
<td>-13.723 (-15.446, -11.999)</td>
<td>-43.228 (-53.879, -32.577)</td>
<td>p &lt; 0.0001 d</td>
<td>p &lt; 0.0001 d</td>
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<tr>
<td>p &lt; 0.0001 d</td>
<td>p &lt; 0.0001 d</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Footnotes for Table 6-5.

a Statistical analysis was performed as reported in Section 2.9.

b The levels of DHBV total and cccDNA in the liver of ducks in Groups 6A vs. 6B, Groups 6A vs. 6C and Groups 6B vs. 6C were not significantly \( (p = 0.9950 - 1) \) different at day 14 and 63 p.i. reflecting the DHBV surface or core antigen or a combination of two was equally effective as components of our post-exposure “prime-boost” protocol.

c The levels of DHBV total and cccDNA in the liver of ducks in Group 6A vs. 6D, Group 6B vs. 6D and Group 6C vs. 6D were not significantly different \( (p = 0.9986 – 0.9997) \) at day 14 p.i. due to the effect of ETV treatment on reducing the spread of DHBV infection, but was more statistically significant \( (p = 0.1283 – 0.2985) \) at day 63 p.i. reflecting the rebound of the DHBV infection in Group 6D.

d Differences in the levels of liver DHBV total and cccDNA between the ducks in Group 6D vs. 6E were highly statistically significant \( (p < 0.0001) \) at all time-points.
Figure 6.6. The mean levels of DHBV total and cccDNA in the liver of ducks in Groups 6A, 6B, 6C, 6D and 6E on days 14 and 63 p.i.

Twenty-five 14-day-old ducks were inoculated i.v. with $5 \times 10^8$ DHBV genomes (Pool 8) and were vaccinated as described in the legend to Figure 6.1.

Total cellular and viral DNA was extracted from biopsy and autopsy liver tissues as described in Section 2.8.2 and subjected to qPCR analysis for levels of DHBV total and cccDNA using primer sets 423-576c and CC2-MG1 (Table 2-6) as described in Section 2.8.5. The limit of detection of the assay shown as the dotted line in the Figure is determined by the limit of sensitivity of the qPCR machine which does not accurately detect less than 10 copies of total DHBV DNA per 53,571 cells, or 0.00019 copies of DHBV DNA per cell.

Upper Panel: Mean levels of DHBV total DNA in the liver of ducks in Groups 6A, 6B, 6C, 6D and 6E on days 14 and 63 p.i.

Lower Panel: Mean levels of DHBV cccDNA in the liver of ducks in Groups 6A, 6B, 6C, 6D and 6E on days 14 and 63 p.i.

Group 6A: ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C and rFPV strains, rFPV-DHBpre-S/S and rFPV-DHBc.

Group 6B: ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S and pcDNA3-S and rFPV strain, rFPV-DHBpre-S/S.

Group 6C: ducks received ETV, DHBV DNA vaccine pcDNA3-C, and rFPV strain, rFPV-DHBc.

Group 6D: ducks received ETV and control vectors (pcDNA3 and FPV-M3).

Group 6E: ducks received water and control vectors (pcDNA3 and FPV-M3).
6B on day 14 and 63 p.i. This suggests that all 5/5 Group 6B ducks had cleared their DHBV infection.

Interestingly, following DHBV infection and vaccination, the levels of anti-DHBs antibodies in Group 6B were lower than the two ducks in Group 6A (Figure 6.3 A-B). In duck 6A.1, anti-DHBs antibodies levels were detected starting from day 28 (14 p.i.) and were 5 times higher than Group 6B ducks. It was proposed that virus particles contain both surface and core proteins, priming of the humoral response to core (e.g. activation of the core specific CD4+helper T lymphocytes) could also accelerate the humoral response to surface, leading to robust anti-DHBs antibody responses in ducks 6A.1 and 6A.5 that were vaccinated with DHBV DNA vaccines expressing both surface and core. The data obtained from Group 6B corroborates the previous reports on HBcAg specific helper T lymphocytes have the unique ability to sustain anti-HBs antibody production (Milich et al., 1987; Lobaina et al., 2005; Akbar et al., 2012). However, it is acknowledged that only two out of the five Group 6A ducks showed higher anti-DHBs antibody responses compared to Group 6B and thus this observation may need further confirmation. Therefore, whether the DHBcAg specific T-cell responses can provide help to B lymphocytes to induce specific antibody against DHBV surface antigens is not known and requires further investigation.

In addition, ETV treated and “prime-boost” vaccinated ducks in Group 6B (ETV + surface) when challenged with DHBV developed anti-DHBc antibodies slower compared to ducks in Group 6A (ETV + surface + core) and 6C (ETV + core). As shown in Figure 6.4, two out of five ducks, ducks 6A.2 and 6A.3 from Group 6A and all five ducks from Group 6C had anti-DHBc antibodies in the serum detected from day 28 (14 p.i.) that persisted until the end of the experiment. In contrast, in Group 6B, anti-DHBc antibodies were only detected starting from day 35 (21 p.i.), one week after ETV treatment was withdrawn. The early development of anti-DHBc antibodies in Group 6A and 6C ducks could be solely due to the effect of core
vaccination not from virus infection since virus replication was still inhibited by ETV on day 28 (14 p.i.).

6.3.3 Group 6C: DHBV infection outcomes in ducks that received ETV treatment in combination of “prime-boost” vaccines that expressed DHBV core antigens.

On biopsy at day 14 p.i., analysis of the liver tissue revealed that DHBV infection was found in ducks 6C.1 (0.01% of DHBsAg-positive hepatocytes) and 6C.5 (0.006% of DHBsAg-positive and 0.004% of DHBcAg-positive hepatocytes) by immuno-staining (Table 6-2). The remaining three ducks had no detectable (<0.001%) DHBV-positive hepatocytes (Table 6-2). This indicated that in these 2 ETV-treated ducks vaccination with core antigen alone was not able to restrict virus infection and induce immune responses to target DHBV-infected hepatocytes compared to ETV treated ducks vaccinated with surface antigens (Group 6A and 6B) by day 14 p.i. This observation is not a surprise since the DHBV DNA vaccines expressing core antigen in Group 6C induce non-neutralising anti-DHBc antibodies which confer no protection against DHBV infection, unlike the neutralising anti-DHBs antibodies induced by DHBV DNA vaccines expressing surface antigens in Group 6A and 6B ducks that can bind to the viruses to prevent infection of the liver (Kuroki et al., 1995; Vickery et al., 1989; Jilbert and Kotlarski, 2000).

The livers were again analysed at autopsy on day 63 p.i., 49 days after withdrawal of ETV, to allow assessment of the outcome of DHBV infection. At this time, DHBV-infected hepatocytes were no longer detected (<0.001%) in all 5/5 Group 6C ducks (Table 6-2), indicating that all 5/5 Group 6C ducks had cleared their DHBV infection and that vaccination had prevented the development persistent DHBV infection. This was further confirmed by detection of low levels of DHBV total (Range: 0.0007-0.04 copies/cell) and cccDNA (Range: 0.0003-0.0017 using qPCR (Figure 6.5 C). These low levels of DHBV total and
cccDNA are not unexpected as similar levels of residual DHBV DNA have been detected in ducks that had cleared their acute DHBV infection (Le Mire et al., 2005; Miller et al., 2008; Reaiche et al., 2010; Low, Ph.D. thesis, 2012).

Analysis of DHBsAg levels in the serum following DHBV infection and vaccination again highlighted the effectiveness of ETV treatment combined with the “prime-boost” vaccination regime. Group 6C ducks had low levels of serum DHBsAg (duck 6C.4 had the highest DHBsAg of 0.66 µg/mL detected at day 70 (Figure 6.2 C). In contrast, high levels of serum DHBsAg persisted throughout the experiment in four out of the five Group 6D (ETV + control vector) and all 5/5 Group 6E (water + control vector) ducks, reflecting the extensive DHBV infection in the liver (Figure 6.2 E).

Analysis of antibody responses by ELISA showed that Group 6C ducks rapidly developed high titres of anti-DHBc antibodies following DHBV infection and vaccination (Figure 6.4 C) compared to Group 6B (ETV + surface alone) ducks (Figure 6.4 B). In contrast, all Group 6C ducks did not produce detectable levels of anti-DHBs antibodies in the serum during the time course of experiment (Figure 6.3 C). Similar to HBV infection, the neutralising anti-DHBs antibodies is a marker of resolution and can bind to the viruses to prevent infection of the liver (Jilbert and Kotlarski, 2000; Kuroki et al., 1995; Vickery et al., 1989). In Group 6C ducks, the neutralising anti-DHBs antibodies may form immune complex with the infectious virions or particles. Therefore, only low levels of circulating anti-DHBs antibodies may present in the serum and the presence of the immune complex may not be detected in our ELISA test.

During resolution of HBV infection, vigorous HBcAg specific T-cell responses are detected in patients but is predominantly absent in patients with CHB (Penna et al., 1996; Guidotti et al., 1999; Thimme et al., 2003). Therefore, we hypothesised that vaccines expressing DHBV
core antigens might be crucial to achieve sustained antiviral immune responses and led to the clearance of DHBV infection. In this study, since neutralising anti-DHBs antibodies were not detected at any time in Group 6C ducks and anti-DHBc antibodies provide no protection against DHBV infection, this indirectly demonstrated the ability of the “prime-boost” vaccination expressing core antigen on induction of CMI responses that target infected hepatocytes to prevent the development of persistent DHBV infection.

In summary, all 5/5 Group C ducks had cleared their DHBV infection and the data indirectly showed that CMI responses against DHBV core might be crucial during the resolution of DHBV infection.

6.3.4 Group 6D: DHBV infection outcomes in ducks that received ETV treatment and control vectors.

At biopsy on day 14 p.i., DHBsAg (Figure 6.2 D) and anti-DHBs antibodies (Figure 6.3 D) were not detected in the serum during the course of ETV treatment. Analysis of the liver tissue revealed that all 5/5 Group 6D ducks that received ETV treatment for 14 days had a reduced percentage of DHBsAg-positive hepatocytes (Range: 0.02-0.45%) and DHBcAg-positive hepatocytes (Range: <0.001-0.75%) compared to all 5/5 Group 6E (water + control vector) ducks that showed >95% of DHBsAg-positive (and DHBcAg-positive) hepatocytes in the liver at day 14 p.i. (Table 6-2) (P <0.0001, Table 6-3 and Table 6-4).

In the study from Foster et al., 2005, ETV treatment for 49 days did not prevent initial infection of the liver but ETV suppressed DHBV replication ~ 10,000-fold. In agreement with the study (Foster et al., 2005), Group 6D ducks had lower levels of total DHBV DNA (Range: 0.0028-0.074 copies/cell) than Group 6E (water + control vector) ducks (Range: 22.6-103.6 copies/cell) at day 14 p.i. (Figure 6.5 E-D) ((P <0.0001, Table 6-5). Similarly, cccDNA levels
were lower in Group 6D (Range: 0.003-26.64 copies/cell) than Group 6E (Range: 35.6 -50.9 copies/cell) at day 14 p.i. (Figure 6.5 E-D) (P <0.0001, Table 6-5). The results again indicated that ETV treatment in Group 6D decreased the DHBV DNA about 10,000-fold when compared to Group 6E, and did not prevent initial virus infection but restricted virus spread in the liver.

At autopsy on day 63 p.i., anti-DHBs antibodies were not detected (Figure 6.3 D) but DHBsAg-positive hepatocytes (Range: 0.15->95%) and DHBcAg-positive hepatocytes (Range: 0.19->95%) (Table 6-2) were detected in four out of five Group 6D ducks, except duck 6D.4. In addition, higher levels of serum DHBsAg (Figure 6.2 D) was also detected in these Group 6D ducks (except duck 6D.4), indicating that DHBV infection had rebounded in these ducks 49 days after ETV treatment was withdrawn. This finding was further supported by qPCR analysis of liver samples for DHBV total and cccDNA, that showed an increase of total DHBV DNA levels (Range: 0.1-69.16 copies/cell) and cccDNA levels (range: 0.003-26.637 copies/cell) (Figure 6.5 E-D) in these four Group 6D ducks. In particular, duck 6D.1, that had the highest levels of DHBV total and cccDNA (69.1 and 26.6 copies/cell) in the liver at day 28 (14 p.i.) compared to other ETV-treated ducks in Groups 6A, 6B, 6C and 6D (Figure 6.5A-D), had developed widespread DHBV infection at day 77 (63 p.i.), with >95% of DHBsAg-positive hepatocytes in the liver, reflecting persistent DHBV infection was established in this duck after the ETV was withdrawn.

In contrast, on day 63 p.i., 49 days after ETV treatment was withdrawn, the remaining duck in Group 6D, duck 6D.4, had undetectable DHBV-positive hepatocytes (or <0.001%) (Table 6-2) and serum DHBsAg (Figure 6.2 D) with transient detection in anti-DHBs antibodies (Figure 6.3 D) on day 49 (35 p.i.) and 77 (63 p.i.), indicating duck 6D.4 had recovered from DHBV infection. In addition, levels of DHBV total and cccDNA were maintained at a low level (0.0025 and 0.0003 copies/cell respectively) on day 63 p.i. (Figure 6.5 D). This
observation was similar to our previous studies (Foster et al., 2005), where only one out of the five 14-day-old ducks given the same dose of the virus in this study and received ETV treatment for 14 days had cleared DHBV infection from the liver and the remaining four ETV-treated ducks showed rebound of virus replication after ETV treatment was withdrawn.

The findings in the Group 6D ducks again validated previous results (Foster et al., 2005; Miller et al., 2008; Feng et al., 2010), showing that short-term ETV treatment for 14 days restricted the spread of the virus within the liver and provided opportunity for the immune response to successfully control DHBV infection in 50% of the 14-day-old ducks infected with 5x10⁸ DHBV genomes. The findings also indicate that antiviral treatment alone is not sufficient to achieve a therapeutic outcome unless additional immune stimulation is provided.

Interestingly, all ETV treated and "prime-boost" vaccination ducks in Groups 6A, 6B and 6C developed anti-DHBc antibodies slower than control ducks from Group 6D. In Group 6D, two ducks, ducks 6D.2 and 6D.5, developed anti-DHBc antibodies rapidly started from day 21 (7 p.i.), a week after virus inoculation (Figure 6.4 D). This indicated that the antiviral effects of ETV treatment combined with "prime-boost" vaccination in Groups 6A, 6B and 6C ducks was more efficient in inhibiting the virus replication, resulting the early development of anti-DHBc antibodies in the Group 6D ducks that were treated with ETV.

### 6.3.5 Group 6E: DHBV infection outcomes of ducks received water treatment and control vectors.

As expected, at day 14 and 63 p.i., all five out of five Group 6E ducks had >95% of DHBsAg-positive (and DHBcAg-positive) hepatocytes in the liver, indicating persistent DHBV infection had developed in this group (Table 6-2). Representative liver samples from one duck in each group can be seen in Figure 6.7.
Figure 6.7. The percentage of DHBsAg-positive hepatocytes in a representative duck from Groups 6A, 6B, 6C, 6D, and 6E on days 14 and 63 p.i.

Twenty-five 14-day-old ducks were inoculated i.v. with 5x10^8 DHBV genomes (Pool 8) and were vaccinated as described in the legend to Figure 6.1.

The percentage of DHBsAg-positive hepatocytes was determined by immuno-staining of EAA-fixed liver tissues with anti-pre-S/S monoclonal antibodies (1H.1) (Pugh et al., 1995) as described in the Section 2.7.1. All the sections were photographed using 200 x magnification and sections were counterstained with haematoxylin. Magnification bar = 100 µm. The arrows indicate the DHBsAg-positive hepatocytes (brown staining) in the cytoplasm of infected hepatocytes. The minimum sensitivity of detection of DHBsAg-positive hepatocytes is <0.001%, based on counting 100,000 hepatocytes in sections of liver tissue. Counts were performed using an eyepiece graticule with 250 x 250 µm grid at 200 x magnification.

Panel A: Group 6A ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C and rFPV strains, rFPV-DHBpre-S/S and rFPV-DHBc.
Panel B: Group 6B ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S and pcDNA3-S and rFPV strain, rFPV-DHBpre-S/S.
Panel C: Group 6C ducks received ETV, DHBV DNA vaccine pcDNA3-C, and rFPV strain, rFPV-DHBc.
Panel D: Group 6D ducks received ETV and control vectors (pcDNA3 and FPV-M3).
Panel E: Group 6E ducks received water and control vectors (pcDNA3 and FPV-M3).
Figure 6.8. The percentage of DHBcAg-positive hepatocytes in a representative duck from Groups 6A, 6B, 6C, 6D, and 6E on day 14 and 63 p.i.

Twenty-five 14-day-old ducks were inoculated i.v. with $5 \times 10^8$ DHBV genomes (Pool 8) and were vaccinated as described in the legend to Figure 6.1.

The percentage of DHBcAg-positive hepatocytes was determined by immuno-staining of formalin-fixed liver tissues with antigen retrieval using polyclonal anti-DHBc antibodies (CQT-2) as described in the Section 2.7.2. All the sections were photographed using 200 x magnification and sections were counterstained with haematoxylin. Magnification bar = 100 µm. The arrows indicate the DHBcAg-positive hepatocytes (brown staining) in the cytoplasm of infected hepatocytes. The minimum sensitivity of detection of DHBcAg-positive hepatocytes is <0.001%. Counts were performed using an eyepiece graticule with 250 x 250 µm grid at 200 x magnification.

**Panel A:** Group 6A ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S, pcDNA3-S, and pcDNA3-C and rFPV strains, rFPV-DHBpre-S/S and rFPV-DHBc.

**Panel B:** Group 6B ducks received ETV, DHBV DNA vaccines, pcDNA3-pre-S/S and pcDNA3-S and rFPV strain, rFPV-DHBpre-S/S.

**Panel C:** Group 6C ducks received ETV, DHBV DNA vaccine pcDNA3-C, and rFPV strain, rFPV-DHBc.

**Panel D:** Group 6D ducks received ETV and control vectors (pcDNA3 and FPV-M3).

**Panel E:** Group 6E ducks received water and control vectors (pcDNA3 and FPV-M3).
The qPCR detection of DHBV total and cccDNA in the liver confirmed the immuno-staining results. At biopsy on day 14 p.i., all 5/5 Group 6E ducks had high levels of DHBV total and cccDNA in the livers (Range: 22.6-104 copies/cell and 11.4-18.07 copies/cell respectively) and the level maintained to day 63 p.i. (Range: 58.6-108.6 copies/cell and 35.6-69.8 copies/cell respectively), indicating that active virus replication was occurring within the liver throughout the experiment (Figure 6.5).

Infection of the liver also resulted in the appearance in the serum of DHBsAg (Range: 0.5 to 40 µg/mL; Figure 6.2 E) and anti-DHBc antibodies (Range: 1147 to 62500 titres; Figure 6.4 E) in the serum without the detection of anti-DHBs antibodies. The low and transient rise of anti-DHBs antibodies in ducks 6E.1 and 6E.4 on day 77 (63 p.i.) could be due to the non-specific binding of ELISA (Figure 6.3 E).

6.4 Discussion:

Patients with CHB usually exhibit immune tolerance, resulting host immune responses to the virus are minimal (Yim and Lok, 2006; Kwon and Lok, 2011). On the other hand, in patients resolving their acute HBV infection, a reduction in viral load precedes the strong and robust T-cell functions that lead to the clearance of infection (Manno et al., 2004; Kara et al., 2004). Based on these observations, in this vaccination protocol, ETV antiviral treatment is used to lower the virus and antigen loads to allow the induction of immune responses to suppress virus replication. Subsequently, the “prime-boost” vaccination protocol is used to restore specific humoral and CMI responses that target infected hepatocytes, which contain the RI DNA and cccDNA. This eventually leads to the clearance of DHBV infection.
We previously showed that post-exposure treatment with ETV treatment from the time of DHBV infection for either 14 or 49 days reduced the spread of infection, with ~1000-fold fewer DHBV-infected hepatocytes by day 7 p.i. (Foster et al., 2005). In the same study, short-term ETV treatment for 14 days provided opportunity for the immune response to successfully control DHBV infection in 50% of the 14-day-old ducks infected with 5x10^8 DHBV genomes, as compared to 100% of untreated ducks. The result suggested that ETV treatment was only able to alter outcome of DHBV infection in ~50% of ducks, indicating that ETV treatment alone is not sufficient to achieve a therapeutic outcome unless additional immune stimulation is provided. Similarly, in the current study, 14 day-old-ducks treated with ETV following infection with a dose of 5x10^8 DHBV genomes showed restricted spread of DHBV infection in the liver and persistent DHBV infection occurred in only 20% of ducks in Group 6D on day 63 p.i. (Table 6-2).

In Group 6D, ETV treatment for 14 days commencing at the same time of DHBV infection resulted in different infection outcomes, with low-levels of DHBV-infected hepatocytes and significant delay in the development of markers of virus replication on day 14 p.i. However, only one out of the five Group 6D ducks, duck 6D.4, developed anti-DHBs antibodies (Figure 6.3) with the remainder showing rebound in all markers of virus replication after ETV treatment was withdrawn. In particular, the mean levels of DHBV total and cccDNA in Group 6D were increased at least ~1000-fold from day 14 p.i. to 63 p.i., 49 days after ETV treatment was withdrawn (Figure 6.6). This is not surprising as ETV does not eliminate the intranuclear cccDNA form (Reaiche et al., 2010).

Similar to HBV infection in humans, the resolution of an acute DHBV infection is characterised by loss of serum DHBsAg and the seroconversion of the DHBsAg to anti-DHBs antibodies (Jilbert et al., 1998; Foster et al., 2003; Foster et al., 2005; Le Mire et al., 2005;
Miller et al., 2008; Feng et al., 2010; Noordeen et al., 2013). From here we inferred that in these ETV-treated ducks, humoral and CMI responses were not well developed, resulting in a rapid rebound in DHBV infection after ETV treatment is discontinued. This is similar to the experience during CHB, in which immune response against CHB is defective and thus withdrawal of the antiviral treatment results in the rebound of HBV infection (Lai et al., 2003; Werle-Lapostolle et al., 2004; Zoulim, 2004; Wursthorn et al., 2006; Yim and Lok, 2006; Xu and Chen, 2006). Taken together, the findings again suggested that if therapeutic outcomes are to be achieved, additional strategies designed to stimulate effective immune responses are needed to improve the likelihood of viral clearance.

In our most recent studies (Miller et al., 2008; Feng et al., 2010), we combined ETV treatment with “prime-boost” vaccines expressing surface and core antigens to a concurrent DHBV infection. This vaccination protocol resulted in removal of DHBV-infected hepatocytes and prevented the development of persistent DHBV infection in 100% of vaccinated ducks. Previous studies in HBV infection showed that HBV surface and core specific CD8+ T lymphocytes are both detected in patients with acute HBV infection but are not detectable in patients with CHB (Penna et al., 1996; Webster et al., 2004). Therefore, as the next step toward developing a potential therapeutic DHBV vaccine, the experimental protocols in this study were designed to determine if DHBV surface antigen which generates neutralising anti-DHBs antibodies, or DHBV core antigen which generates non-neutralising anti-DHBc antibodies, could provide the essential epitopes in a DNA vaccine and “prime-boost” protocol to enable the resolution of DHBV infection.

In this current study, the development of DHBV infection was prevented by day 14 p.i. in all Groups 6A (ETV + surface + core) and 6B ducks (ETV + surface) as well as 3 out of 5 Group 6C (ETV + core) ducks (Table 6-2) as there were no DHBsAg-positive (and DHBcAg-
positive) hepatocytes detected in the liver of ducks by immuno-staining. The direct data showed that ducks that received ETV treatment in combination with “prime-boost” vaccination expressing DHBV surface antigens, either in the presence (Group 6A) or absence (Group 6B) of core antigens showed better efficiency than ducks vaccinated with DHBV core antigen alone (Group 6C) at reducing the number of DHBV-infected hepatocytes on day 14 p.i. However, at the end of the experiment, no difference was observed between ETV treated ducks that received “prime-boost” vaccination with either DHBV surface or core antigens, DHBV-infected hepatocytes were no longer detected (<0.001%) on day 63 p.i. (Table 6-2). This indicated that all Groups 6A, 6B and 6C ducks had cleared the acute DHBV infection by day 63 p.i. but they have not cleared the infection completely. The outcome is clearly an effect of the ETV treatment combined with “prime-boost” vaccination. This is because ETV treatment alone did not induce sustained immune responses to suppress virus replication when ETV treatment was discontinued in the control ducks in Group 6D. Therefore, the data suggests that ETV treatment combined with “prime-boost” vaccination not just inhibit virus replication and spread, but also stimulate specific immune responses to target DHBV-infected hepatocytes leading to the successful control of DHBV infection.

Numerous studies in humans, woodchucks and ducks showed that the residual viral DNA, in particular the cccDNA in the livers, is responsible for the persistent infection and reactivation of virus replication (Mason et al., 1994; Michalak et al., 1994; Newbold et al., 1995; Yotsuyanagi et al., 1998; Michalak et al., 1999; Le Mire et al., 2005, Reaiche et al., 2010). It is therefore essential to investigate the therapeutic effect of novel vaccination protocols on clearance of infected hepatocytes and the elimination of liver DHBV total and cccDNA. In this study, all 15 ducks treated with ETV and “prime-boost” vaccination in Group 6A, 6B and 6C had cleared DHBV infection by day 63 p.i. as DHBsAg-positive (and DHBcAg-positive) hepatocytes were no longer detected in the liver by immuno-staining. It was also interesting to note that residual DHBV DNA was detected by qPCR analysis of liver DNA extracts in
these 15 ducks, except duck 6A.3 on day 63 p.i. (Figure 6.5). In patients resolving their acute HBV infection, residual HBV DNA can also be observed in the livers despite the presence of anti-HBs antibodies (Yuki et al., 2003). Thus, these low levels of residual DNA are not unexpected in here as similar levels of residual DHBV DNA have been detected in ducks that had cleared their acute DHBV infection (Le Mire et al., 2005; Miller et al., 2008; Reaiche et al., 2010; Low, Ph.D. thesis, 2012).

In addition, the mean levels of DHBV total and cccDNA in Groups 6A, 6B and 6C were at least 500-fold lower than DHBV-infected and untreated ducks in Group 6E (Figure 6.6). Again, the data suggests that our ETV treatment in combination with “prime-boost” vaccination protocol is efficient in inhibiting virus replication while targeting destruction of DHBV-infected hepatocytes. Although there is variation from duck-to-duck, no major difference in levels of residual DHBV DNA were noted among individual ETV treatment groups in combination with “prime-boost” vaccines that expressed DHBV surface, core or both antigens. These findings indicate that DHBV surface and core antigen as measured in our studies are equally effective as components of our post-exposure “prime-boost” protocol. However, we must acknowledge that the small number of animals used in each group were not sufficient to determine statistical significance.

After the rFPV-DHBV vaccines boost on day 7 p.i. and ETV treatment was withdrawn on day 14 p.i., during 49 days of follow-up, the serum DHBsAg remained low or undetectable in Groups 6A, 6B and 6C ducks (Figure 6.2). The absence of DHBsAg-positive (and DHBcAg-positive) hepatocytes in the liver tissues collected on day 63 p.i. again confirmed the clearance of DHBV infection in Groups 6A, 6B and 6C ducks. In contrast, 4 out of 5 Group 6D ducks (ETV + control vector) and 5 out of 5 Group 6E ducks (water + control vector) had detectable levels of serum DHBsAg (Figure 6.2) and DHBsAg-positive (and DHBcAg-positive) hepatocytes in the liver (Table 6-2), suggesting active virus replication in these
ducks. In many studies using duck model, rebound in DHBV replication was observed after NAs treatment withdrawal (Le Guerhier et al., 2003; Foster et al., 2003; Foster et al., 2005; Miller et al., 2008; Feng et al., 2010). In this study, the antiviral effect of our vaccination protocol was sustained since no rebound of virus replication was observed 49 days after the end of therapy. This again highlighted the superior therapeutic effect of the ETV treatment combined with “prime-boost” vaccination over antiviral treatment alone.

As mentioned earlier, the resolution of an acute DHBV infection is characterised by loss of serum DHBsAg and the seroconversion of the DHBsAg to anti-DHBs antibodies (Jilbert et al., 1998; Foster et al., 2003; Foster et al., 2005; Le Mire et al., 2005; Miller et al., 2008; Feng et al., 2010; Noordeen et al., 2013). The neutralising anti-DHBs antibodies that can bind to the viruses to prevent infection of the liver are also a marker of resolution of an acute DHBV infection (Kuroki et al., 1995; Vickery et al., 1989; Jilbert and Kotlarski, 2000). At the end of the experiment on day 63 p.i., all 15 Groups 6A, 6B and 6C ducks had detectable anti-DHBc antibody responses (Figure 6.4) while anti-DHBs antibody responses were only detected in 4 out of 5 Group 6A (ETV + surface and core) and 3 out of 5 Group 6B (ETV + surface) ducks (Figure 6.3). All five Group 6C ducks (ETV + core) had no detectable anti-DHBs antibodies in the serum throughout the study. These findings suggest that protective anti-DHBs antibody responses may be responsible for the early protection observed in the Group 6A and 6B ducks that treated with ETV and vaccinated with DHBV surface antigens, by preventing the initial infection or re-infection and also induce significant CMI to remove DHBV-infected hepatocytes. On the other hand, the failure to detect anti-DHBs antibody responses in the remaining ducks in Groups 6A, 6B and 6C ducks that had cleared DHBV infection by day 63 p.i. could be due to the formation of immune complex that could not be detected in our ELISA assay.
It has been demonstrated that “prime-boost” vaccination in the absence of the antiviral treatments enhanced the T-cell mediated immune responses, particularly the Th1 response that is characterised by the production of IFN-γ (Kent et al., 1998; Walther et al., 2006; Rerks-Ngarm et al., 2009). In the current study, it is believed that other than the humoral immune responses, the elimination of DHBV infected hepatocytes primarily as the result of targeting by the CMI responses generated by “prime-boost” vaccination strategies as DHBV infection itself is non-cytopathic.

Immune responses to DHBV core antigens will generate non-neutralising anti-DHBc antibodies which confer no protection against DHBV infection, unlike the neutralising anti-DHBs antibodies (Kuroki et al., 1995; Vickery et al., 1989; Jilbert and Kotlarski, 2000; Gares et al., 2006). In Group 6C ducks that treated with ETV and vaccinated with core antigens alone, the absence of a detectable anti-DHBs antibody response (Figure 6.3) indicated that an antibody independent response played a key role in the clearance of DHBV-infection. Therefore, the non-neutralising anti-DHBc antibody response may account for the lower protective efficacy in 2 out of the five Group 6C ducks at day 14 p.i. (Table 6-2) but this was later overcome by the CMI responses induced by core antigens. At day 14 and 63 p.i., the lower levels of mean DHBV total and cccDNA in Group 6C ducks compared to Groups 6E ducks (Figure 6.6) had indirectly showed that core antigen specific CMI responses might be responsible for the viral clearance since the destruction of DHBV-infected hepatocytes is the most efficient way to eliminate cccDNA. Ideally we would like to measure specific CMI in ducks to confirm our observation. However, due to the lack of duck specific reagents to detect CMI responses in ducks, the overall therapeutic effect of our ETV treatment combined with “prime-boost” vaccination protocol cannot be assessed.
Our results demonstrate that protective immune responses generated by “prime-boost” vaccination strategies with either DHBV surface or DHBV core alone blocked virus spread and replication. Importantly, the overall decrease in mean DHBV total and cccDNA levels (Figure 6.6) and percentage of DHBV-infected hepatocytes (Table 6-2) in Groups 6A, 6B and 6C ducks compared to Groups 6D and 6E on day 63 p.i. again indicate that DHBV surface and core antigen as measured in our studies are equally effective as components of our post-exposure “prime-boost” protocol.

A published study of patients with acute HBV infection showed that CD4+ T lymphocyte responses can be stimulated by multiple epitopes within HBV nucleocapsid protein; with 90% tested patients had HBV specific CD4+ T cell responses to identified immuno-dominant core epitope (Ferrari et al., 1991). At the immune control stage of persistent HBV infection, increased core-specific CD4+ T cell responses are detected followed by seroconversion of HBeAg to anti-HBe antibodies, which implies the reduced levels of virus replication (Bertoletti et al., 2006; Tsai et al., 1992). This suggests nucleocapsid specific CD4+ T cell responses might be the dominant helper responses in resolution of HBV infection. In contrast, HBV envelope protein stimulates weaker CD4+ T cell responses compared to core antigen during HBV infection, which might due to differences in antigen presentation (Bertoletti et al., 2006). Recently, HBV polymerase specific CD4+ T cell responses have been demonstrated more frequently in patients with acute HBV infection or in patients after recovery from CHB. In addition, transient increases in HBV polymerase specific CD4+ T cell responses with reduced levels of virus replication were observed in patients with CHB (Mizukoshi et al., 2004). This indicates that HBV polymerase epitopes could be a valuable component of therapeutic HBV vaccines.

In addition, it is known that the HBV specific CD8+ T cell response is stronger in patients with acute HBV infection than CHB and those responses persist for decades after resolution.
from HBV infection (Bertoletti et al., 2006). Studies in patients with acute and persistent HBV infection also revealed that levels of virus replication alter the epitope hierarchy of the HBV specific CD8+ T lymphocyte population. The study showed that HBV core specific CD8+ T lymphocytes are immuno-dominant in patients with acute HBV infection but are not detectable in patients with CHB. This indicates that the frequency of HBV core specific CD8+ T lymphocytes is inversely proportional to levels of viral replication (Webster et al., 2004). In contrast, HBV envelope and polymerase specific CD8+ T lymphocytes are detectable in patients with CHB infection even when the levels of HBV replication are high. In patients with CHB, the magnitude of the HBV envelope-specific CD8+ T lymphocyte response remained unchanged regardless the levels of virus replication. This suggests that HBV envelope specific CD8+ T lymphocytes are non-responsive or anergic (Reignat et al., 2002). On the other hand, lower levels of polymerase antigens expressed on infected hepatocytes compared to other HBV antigens might limit the antiviral activity of HBV polymerase-specific CD8+ T lymphocytes (Kakimi et al., 2002; Mizukoshi et al., 2004).

As described above, HBV specific CD4+ and CD8+ T lymphocytes are able to be stimulated by various epitopes within the HBV nucleocapsid, envelope and polymerase protein. Therefore, it is believed that multiple vaccine antigens can induce broader and more efficient humoral and CMI responses. The results of this study demonstrated that DHBV core or surface alone is sufficient to confer protective immune responses to target DHBV-infected hepatocytes and prevent the development of widespread and persistent DHBV infection. However, ducks treated with ETV plus “prime-boost” vaccines expressing both DHBV core and surface antigens (Group 6A) had better efficacy in elicit protective anti-DHBs antibodies and targeting DHBV-infected hepatocytes that led to the suppression of virus replication. Therefore, it is believed multiple vaccine antigens (DHBV core and surface antigens) are essential in our current combination antiviral therapy and “prime-boost” vaccination protocol.
to induce broader and better efficient humoral and CMI responses for future therapies for persistent DHBV infection.

In summary, the current study underlines the importance of our ETV plus “prime-boost” vaccination protocol in lowering viral load and inducing antiviral immune responses, which target the DHBV-infected hepatocytes and prevent the development of persistent DHBV infection. More importantly, the antiviral effect of our vaccination protocol was sustained since no rebound of virus replication was observed 49 days after the end of therapy. Our data also suggested that DHBV core alone was not able to induce protective humoral response but induced effective CMI responses that allowed the elimination of DHBV-infected hepatocytes and prevented the development of widespread DHBV-infection. At present, we were unable to investigate whether this vaccination protocol led to the induction of specific CMI responses due to the limitation on duck specific reagents. Overall, the data showed that DHBV surface and core antigen as measured in our studies are equally effective as components of our post-exposure “prime-boost” protocol. In the future, studies that extend the time after DHBV infection to allow a higher percentage of infected hepatocytes at the onset of treatment need to be investigated. Long-term follow up study is also required to investigate the antiviral effect of our vaccination protocol on residual DHBV DNA. Lastly, larger numbers of animals per group are also needed to obtain statically significant data.
Chapter 7: Discussion

7.1 Introduction
Despite the success of HBsAg-containing prophylactic vaccines in preventing human HBV infection, CHB is still a tremendous global health problem. Liver cirrhosis or HCC, a direct consequence from CHB, causes 1 million deaths each year (Grimm et al., 2011). As mentioned in Chapter 1, current approved antiviral therapies for CHB with either IFN-α or monotherapy with NAs such as 3TC, TFV, ADV and ETV are not highly effective due to the persistence of cccDNA in hepatocytes and the emergence of drug resistant viruses. Therefore, the questions for managing CHB still exist: Can we develop more effective therapeutic strategies to treat CHB, such as therapeutic vaccines? Given that HBV has different antigens, what is the best combination of antigens for constructing these therapeutic vaccines?

7.2 The optimisation of methods
DHBV-infected ducks and WHV-infected woodchucks are the most common animal models for the study of hepadnavirus infection (Karayiannis, 2003). DHBV infection outcomes are virus dose and host age dependent (Qiao et al., 1990; Vickery and Cossart, 1996; Jilbert et al., 1998; Foster et al., 2005; Miller et al., 2008; Feng et al., 2010; Noordeen et al., 2013), which is similar to that seen in HBV infection in humans allowing DHBV-infected ducks to be a suitable hepadnavirus animal model for monitoring the efficacy of anti-viral treatments and immunotherapies.

DHBV infection related assays have been developed in the HBV Research Laboratory to monitor the kinetics of virus infection and specific immune responses activated by DHBV infection (Miller et al., 2004). These assays include ELISA assays for DHBsAg, anti-DHBs and anti-DHBc antibodies, immuno-staining of DHBsAg-positive hepatocytes using
monoclonal anti-pre-S/S antibodies (1H.1) (Pugh et al., 1995) and detection of DHBV total and cccDNA using qPCR (Reaiche, Ph.D Thesis, 2008; Reaiche et al., 2010; Reaiche-Miller et al., 2013). In the current study, we used DHBV-infected ducks as a model and employed these well established techniques to evaluate the efficacy of our newly developed immunotherapy strategies.

In patients with CHB, the presence of serum HBsAg in conjunction with the detection of HBcAg in liver biopsy samples provide evidence of ongoing virus replication (Gudat et al., 1975; Hadziyannis et al., 1983). In HBV infection, although nucleocapsids composed of HBcAg are predominantly detected in the nuclei of hepatocytes in primates (Arnord et al., 1975; Gudat et al., 1975; Huang et al., 1979; Mason et al., 2009) and transgenic mice (Weber et al., 2001), cytoplasmic HBcAg has been reported previously (Gowans et al., 1985; Kakumu et al., 1989). It was suggested that cytoplasmic HBcAg is a more reliable marker for virus replication in comparison to nuclear HBcAg, as the nuclear HBcAg is not directly involved in virus replication (Gowans et al., 1985).

In contrast, WHV core antigen (WHcAg) (Kajino et al., 1984; Ponzetto et al., 1984; Summers et al., 2003) and DHBcAg (Jilbert et al., 1992) are primarily detected in the cytoplasm of infected hepatocytes and have been used as a reliable marker for active virus replication. Thus, the expression of DHBV core antigen is considered as an important biomarker for evaluating the effect of anti-viral therapies. However, the detection of DHBcAg-positive hepatocytes in duck liver tissue was hampered due to the non-specific staining using the polyclonal anti-DHBcAg-specific antibodies (R39408). One of the aims in the current study was to produce anti-DHBcAg antibodies.

Chapter 3 of this thesis described the production of DHBcAg specific polyclonal and mABs using rabbits and mice immunised with rDHBcAg purified from E. coli. Protocols for
immuno-staining, Western Blotting and IMF detection of DHBcAg using these anti-DHBcAg-specific antibodies were then developed and optimised. These assays were shown to be reliable and sensitive and are now in routine use in the HBV Research Laboratory.

The immuno-staining of DHBsAg-positive hepatocytes with monoclonal anti-pre-S/S antibodies (1H.1) (Pugh et al., 1995) is a sensitive assay which allows detection of as few as 0.001% of infected hepatocytes (Foster et al., 2003, 2005; Miller et al., 2006, 2008; Feng et al., 2010; Reaiche-Miller et al., 2013; Noordeen et al., 2013). Of the assays developed during this study, immuno-staining for the detection of DHBcAg-positive hepatocytes in the liver proved to be as sensitive as the detection of DHBsAg-positive hepatocytes (Chapter 3, Section 3.3.2.4). The results showed that the percentage of DHBsAg- and DHBcAg-positive hepatocytes were 2.2% and 1.9% respectively in liver samples collected from the same DHBV-infected duck at the same time-point. This technique is reliable and the non-specific staining of these anti-DHBcAg-specific polyclonal antibodies was negligible after they were absorbed with NDL and NDS. This indicates that this assay has low percentage of false-positive staining, allowing the comparison with detection of DHBsAg and confirmation of the percentage of DHBV-infected hepatocytes.

In this study, the detection of similar percentage of DHBsAg- and DHBcAg-positive hepatocytes in the same liver also suggests that each hepatocyte is producing both antigens. This contradicts the observation in patients with CHB, showing HBV persistence in the absence of HBsAg or HBcAg or both in the liver (Gudat et al., 1975; Trevisan et al., 1978; Cuccurullo et al., 1987). The discrepancy observed between HBV- and DHBV-infected liver could be due to the longer lifespan of primates compared to ducks, allowing long-term infection that facilitates the integration of HBV DNA into liver genome (Mason et al., 2009, 2010). This integration might lead to the changes in virus and host gene expression, resulting
in the absence of HBV antigen expression in the liver. Nonetheless, this question remains to be elucidated and needs to be addressed in the future studies.

As described in Chapter 3, antibody titres of immune sera from mice that were immunised with plasmid DNA expressing DHBcAg were lower than in mice immunised with rDHBcAg purified from E. coli lysates. This phenomenon could be explained if the delivery of plasmid DNA i.m. resulted in low levels of protein expression in transfected cells in mice. It was previously reported that i.v. DNA immunisation improved the transfection efficiency and high antibody titres were obtained even after single i.v. DNA immunisation (Surovoi et al., 2001). If successful, this proposed method with DNA immunisation would help to avoid the current time-consuming protein purification process and would simplify the procedure of obtaining mABs.

7.3 The use of a CD40L expression construct to improve immunogenicity of DNA vaccines

DNA vaccines have many advantages over conventional vaccines in terms of cost and production speed (Liu, 2003). DNA vaccination has been known to induce the intracellular expression of the cloned antigens that closely resemble native viral antigens vaccines (Beckebaum et al., 2002). This is highly regarded for HBV vaccines and may result in an increase in the immunogenicity of the cloned HBV epitopes. In addition, the current standard HBV vaccine requires the administration of three i.m. doses over ~6 months (WHO, 2011). By incorporating several different viral epitopes into the same plasmid DNA (Beckebaum et al., 2002), DNA vaccination may reduce the dose of the current prophylactic HBV vaccines to achieve protection against HBV infection.
Therapeutic DNA vaccination has been shown to induce humoral and CMI responses that are similar to that observed in acute HBV-infected patients (Loirat et al., 2000). The ability of DNA vaccines to induce specific humoral and CMI responses against HBV was further demonstrated in different models of hepadnavirus infection such as in transgenic mice (Rollier et al., 1999; Xing et al., 2005), woodchucks (Lu et al., 1999) and ducks (Triyatni et al., 1998). Consistent with these studies, the current studies demonstrated that the ability of DNA vaccines to induce specific immune responses as described in Chapter 4 and 5. The ducks vaccinated with DNA vaccines expressing DHBV surface or core antigens and challenged with DHBV genomes at 6 weeks of age (Chapter 4) and at 14 days of age (Chapter 5) induced strong, long lasting and neutralising anti-DHBs antibodies and the ducks were able to resolve DHBV infection. In contrast, all vector vaccinated control ducks in these studies did not induce long lasting anti-DHBs antibodies, failing to clear DHBV infection following virus challenge.

Although DNA vaccines are efficient in inducing specific immune responses in small animals as mentioned above, the immunogenicity of DNA vaccines remains relatively low in large animals (Babiuk et al., 1999) and non-human primates (Kim et al., 2000). Therefore, to translate these research achievements to human clinical trials, the protocols should be optimised. In this study, a DuCD40L expression construct was thus used as a genetic adjuvant to improve the immunogenicity of DNA vaccines. The CD40L expression construct was chosen in this study as part of our therapeutic vaccination protocol because we hypothesised that CD40L enhances the immunogenicity of our DNA vaccines, reaching an optimised therapeutic effect for CHB.

The interaction between CD40L and CD40 mediates the humoral and CMI responses, particularly the T-cell immune responses (Magott-Procelewksa, 2004; Krocze and Hamelmann, 2005). In HBV infection, T-cell responses to HBV are rarely detected in patients
with CHB, and this is thought to be due to T-cell exhaustion or anergy (Rehermann et al., 1995; Boni et al., 2003; Bertoletti et al., 2006). Recently, impaired lymphocyte activation was observed in non-responders to the HBV vaccine, which include a diminished capability to up-regulate CD40L expression (Goncalves et al., 2004). In a more recent study, following stimulation with HBcAg alone or in combination with cross-linking of the CD28 receptor, CD40L levels did not increase in CD4+ T lymphocytes from patients with CHB but did increase in individuals that had acute HBV infection (Barboza et al., 2009). This suggests that impaired T lymphocyte co-stimulation may play an important role in development of immune tolerance to HBV antigens during CHB.

This was further supported by a recent phase I/IIa clinical trial, that used adenoviral vector-based CD40L (AdCD40L) gene therapy vectors to treat patients with invasive bladder cancer. In these studies AdCD40L therapy stimulated infiltration of CD4+ and CD8+ T lymphocytes into bladder tissues (Malmstrom et al., 2010). The study also showed that AdCD40L therapy stimulated Th1 responses and suppressed regulatory T lymphocytes, indicating that CD40L therapy in humans may counteract or redistribute regulatory T lymphocytes. The ability of CD40L in aiding the shift from immunosuppression to immune activation has led to the view that CD40L may be beneficial for the treatment of CHB when used as a single vaccine or as an adjuvant.

Currently, a number of studies involve in the administration of CD40L as an immunological adjuvant in anti-tumor and antiviral therapies. These include: (1) CD40L expressed by plasmid DNA constructs (Stone et al., 2006; Yao et al., 2010), (2) CD40L expressing APCs as immunogens (Von Bergwelt-Baildon et al., 2002), (3) CD40L as a fusion protein (Huang et al., 2004; Gomez et al., 2009) (4) CD40L expressed by poxvirus vectors (Feder-Mengus et al., 2005; Liu et al., 2008) or (5) CD40L expressed by adenoviruses (Peter et al., 2002). At present, clinical data on therapeutic efficacy of CD40L co-administration with DNA vaccines
targeting HBV antigens and virus clearance has not been investigated. In the duck model, 
immunisation with a combination of constructs expressing DHBV core antigen plus 
DuCD40L enhanced immune responses compared to the expression of core alone (Gares et 
al., 2006). However, DHBV challenge experiments were not performed and the enhanced 
effect of DuCD40L on the DNA vaccines for treating the persistently DHBV-infected ducks 
was not determined.

In Chapter 4, we described the initial step in designing a DuCD40L expression construct. 
The cloning and analysis of expression of the DuCD40L were performed in our laboratory by 
Dr Feng Feng. Here, the adjuvant effect of CD40L on DNA vaccines was mirrored by the 
results of the DNA vaccine studies in Chapter 4 and 5 using the DHBV model. Studies as 
described in Chapter 4 demonstrated that vaccination of genetic constructs expressing 
DuCD40L can enhance antibody responses after DNA vaccination presumably by targeting 
virus antigens expressed by the DNA vaccines to APCs following DHBV challenge. In 
Chapter 4, administration of the DuCD40L expression construct and the DHBV DNA 
vaccines in combination resulted in a 10-fold greater anti-DHBs antibody response and a 
significant decrease in the number of DHBV-infected hepatocytes at day 4 p.c. compared to 
ducks that received DHBV DNA vaccines alone. However, it was not determined in these 
studies if the benefit of using CD40L expression construct occurred entirely at the specific 
humoral responses or if specific CMI responses were also generated. The antibody responses 
observed from this work were consistent with a recent report that showed that DuCD40L 
enhances DNA vaccine responses which led to accelerated and augmented core-specific 
antibody responses (Gares et al., 2006).

As described in Chapter 5, a somewhat unexpected observation in a protective DNA vaccine 
study in 14-day-old ducks was that following DHBV challenge, no significant difference in 
the percentage of DHBV-infected hepatocytes or anti-DHBs antibody titres was observed
between ducks receiving DHBV DNA vaccines with DuCD40L expression construct and ducks receiving DHBV DNA vaccines alone. This has made the objective in Chapter 5 difficult to assess. We cannot tell whether there was synergy effect between DuCD40L and DNA vaccines or not.

It is possible that the difference in anti-DHBs antibodies response observed in Chapter 4 and Chapter 5 was related to the dose of DNA vaccines (500 µg and 250 µg each vaccine per dose respectively) given to the ducks to induce immune responses. It was previously reported that the number of APCs in muscle tissue are limited (Hohlfeld and Engel, 1994). Thus, the adjuvant effect of the DuCD40L expression construct on i.m. injection of DNA vaccines may be more robust and effective when higher doses of antigen are administered. Also, the age of ducks at time of DHBV challenge could also affect the adjuvant effect of DuCD40L expression construct on DNA vaccines. In these studies, 42-day-old and 14-day-old ducks were challenged with DHBV in Chapter 4 and Chapter 5 respectively. Thus, the maturity of the duck immune system to present the antigens at the time of virus challenge may also have influenced the responses obtained.

Interestingly, in Chapter 5, two out of five ducks that received the DuCD40L expression construct alone (ducks 5E.2 and 5E.3) did not develop widespread DHBV infection, and one duck (duck 5E.2) cleared DHBV infection from the liver. This observation suggested that the DuCD40L expression construct alone may accelerate the engagement of T lymphocytes and therefore enhance immunity of ducks against DHBV challenge, and then effectively control the development of DHBV infection. In addition, the use of pcDNA3 that contains CpG sequences on the plasmid backbone, could also contribute to the spontaneous virus clearance in these two ducks. The CpG sequences, which are present in bacterial DNA, are potent immuno-modulators (Leitner et al., 1999). It has been shown that the CpG sequence has adjuvant effect on the activation of B lymphocytes, resulting higher antibody and CMI
responses (Krieg et al., 1995; Sato et al., 1996; Bode et al., 2011). As to whether it is the DuCD40L expression construct or the CpG sequence or both confer protection against DHBV infection in these two ducks is currently unknown and this deserves further study.

Currently, the mechanism by which DuCD40L helps to prevent the development of DHBV infection in ducks from **Group 4A, Group 4C** (ducks 4C.5 and 4C.6 only) and **Group 4E** (ducks 4E.4 and 4E.5 only) in Chapter 4 as well as **Group 5E** (ducks 5E.2 and 5E.3 only) in Chapter 5 still remains to be elucidated, but we can hypothesise that it was due to the humoral and CMI responses. The enhanced anti-DHBs antibodies response observed in ducks vaccinated with DuCD40L genetic construct and DNA vaccines (Chapter 4; Group 4A) might be due to engagement of secreted DuCD40L with CD40 on activated DHBsAg-specific B lymphocytes and direct induction of B memory lymphocytes. In addition, interaction between DHBsAg-stimulated B lymphocytes via CD40 with CD40L on activated CD4+ T lymphocytes might also promote activation of DHBsAg-specific B lymphocytes resulting in B lymphocyte differentiation and enhanced anti-DHBs antibody responses. In a reciprocal interaction, interaction of co-stimulatory molecules, for instance, B7 on B lymphocytes and CD28 of CD4+ T lymphocytes results in the activation of T lymphocytes. This leads to proliferation of primed T lymphocytes and cytokines secretion. In mammalian systems, virus infection promotes Th1 development that secretes IL-2 and IFN-γ and TNF-β. These Th1 cytokines in returns lead to activation of CTL responses and might result in the initial reduction of DHBV-infected hepatocytes in these ducks following DHBV challenge. Unfortunately, we are not able to investigate the impact of DuCD40L expression construct co-administered with DHBV DNA vaccines on CTL responses because protocols to study CTL responses in ducks are not available in our laboratory.

Before the translation of DNA mediated immunisation to clinical trials, the safety issues associated with the use of DNA vaccines and CD40L expression constructs need to be
considered. In addition, due to the existence of the potential integration of plasmid DNA into
the host cell genome, risking the activation of oncogenes (Robertson and Griffiths, 2001), the
use of these DNA constructs in primates and humans might be considered unethical.
Nonetheless, the studies described in Chapter 4 clearly showed that combination of the
immunological adjuvant DuCD40L with DHBV DNA vaccines provided a first line of
defense against DHBV infection in ducks. The DuCD40L should be further investigated as
an immunological adjuvant in future vaccine studies in enhancing both the quality and
quantity of the humoral and CMI responses, favoring protection against virus infection and
therapies for the ducks infected with DHBV.

7.4 ETV treatment in combination with “prime-boost” vaccination
strategies

In patients resolving their acute HBV infection, a reduction in viral load precedes the strong
and robust T-cell functions (Manno et al., 2004; Kara et al., 2004). Similarly, in patients
showing HBeAg seroconversion during the immune clearance stage of CHB, a reduction in
viral load is usually observed before the restoration of T-cell function (Kwon and Lok,
2011). Based on this observation, monotherapy with NA such as Lamivudine was explored
in clinical trial studies to reduce the viral load and promote the restoration of T-cell activity
(Boni et al., 2001, 2003). Nonetheless, data concluded that the restoration of T-cell activities
induced by NAs is transient, not sustainable and does not promote HBeAg seroconversion
(Boni et al., 2003).

In animal models, particularly in WHV-infected woodchucks and DHBV-infected ducks, the
therapeutic efficacy of NA treatment combined with vaccines was generally improved with
promising outcomes (Le Guerhier et al., 2003; Menne et al., 2002; Roggendorf et al., 2010).
Interestingly, a study performed by our group using the DHBV model showed that
combination treatment of ETV and DNA vaccines did not provide additional therapeutic effects over ETV alone in ducks with widespread DHBV infection (Foster et al., 2003). Similar treatment outcomes were also observed in clinical trial studies that involved the use of Lamivudine in combination with HBsAg-based vaccines, which did not show satisfactory improvement of immune responses (Dahmen et al., 2002; Horiike et al., 2005; Vandepapeliere et al., 2007). This indicates that combination therapy may not be immunogenic enough for use in a CHB therapeutic protocol and thus new concepts of therapeutic vaccination are needed to overcome HBV persistence.

In this current study, we extended studies of a new immunotherapy protocol in the DHBV model that combined anti-viral ETV treatment with “prime-boost” vaccination that was previously shown to prevent the development of widespread and persistent DHBV infection in 5/5 treated ducks (Miller et al., 2008). Proof-of-concept of this approach has been further demonstrated in Chapter 6. In the current study, ETV treatment combined with “prime-boost” vaccination provides additional, immune-mediated control against the spread of DHBV, and significantly enhanced the rate of elimination of DHBV-infected hepatocytes which eventually prevented the development of persistent DHBV infection.

In human clinical trials, ETV is effective against Lamivudine- (Suzuki et al., 2008) and ADV-resistant HBV (Chang et al., 2006). The superiority of ETV over other NAs could be due to its inhibitory action on all three steps in the HBV replication cycle, from priming, reverse transcription to DNA-dependent DNA synthesis (Xu and Chen, 2006; Ferir et al., 2008; Lai and Yuen, 2008). In this study, the success of our novel vaccination protocol was most likely due to suppression of virus replication initiated shortly after the time of infection with the concomitant stimulation of vaccine-induced humoral and CMI responses. The inclusion of ETV treatment is crucial in this vaccination protocol as it was shown to provide opportunity to induce immune responses to control DHBV infection when the viral load is low (Foster et
al., 2005; Miller et al., 2008; Feng et al., 2010). This was further demonstrated in the current study in Chapter 6, where the infection outcomes were altered in vector control ducks treated with ETV (Group 6D), resulting in the resolution of DHBV infection in one out of five ducks. However, antiviral treatment with ETV is unable to clear cccDNA from the liver (Foster et al., 2003; Reaiche et al., 2010) and virus replication will inevitably rebound following the cessation of therapy (Foster et al., 2003), indicating that additional immune stimulation is required.

Despite the fact that the exact mechanism by which “prime-boost” vaccination generates stronger immune responses than DNA vaccination alone in our experimental settings has not been elucidated, various studies in HIV and malaria infection showed that “prime-boost” vaccination in the absence of the antiviral treatments enhanced the T-cell mediated immune responses, particularly the Th1 response that is characterised by the production of IFN-γ (Kent et al., 1998; Walther et al., 2006; Rerks-Ngarm et al., 2009). In a more recent study using the HBV transgenic mouse and WHV-infected woodchuck models, “prime-boost” vaccination using DNA vaccines and adenoviruses that express WHcAg induced Th1 responses, confirmed by the secretion of IgG2 subtype of the antibodies. The study also showed that “prime-boost” vaccination induced more vigorous and functional CD8+ T-cell response than immunisation with DNA vaccine alone (Kosinska et al., 2012).

Based on these findings, we hypothesise that the DNA vaccine may “prime” the T lymphocytes with receptors of high affinity to the cloned antigens and the subsequent immunisation with rFPV vaccines may then result in a robust “boost” of these antigen-specific memory T lymphocytes, triggering the specific humoral and CMI responses that can target infected hepatocytes and prevent the development persistent virus infection. To date, rFPV vaccines have not been extensively studied, but they have been shown to trigger the CMI responses that result in the clearance of DHBV-infected hepatocytes and prevent the
development of persistent DHBV infection (Feng et al., 2010). Ideally, we would like to measure the T-cell responses in ducks to prove the hypothesis. However, due to the lack of duck specific reagents, these investigations have not been performed in this study.

In Chapter 6, another important observation was that protective immune responses generated by ETV treatment combined with “prime-boost” vaccination with either DHBV surface or core alone was sufficient to block virus spread and replication and to result in the targeting of infected hepatocytes. This indicates that DHBV surface and core antigen as measured in our studies are equally effective as components of our post-exposure “prime-boost” protocol.

To date, all of the highly efficacious HBV prophylactic vaccines use HBsAg and the resulting anti-HBs antibodies confer protection against future infection (Mast et al., 2005). The anti-HBs antibodies play a key role in neutralising free virus particles and can prevent (re)infection in non-infected hepatocytes (Akbar et al., 1999). However, HBsAg alone as a therapeutic HBV vaccine may not be effective since HBsAg specific CD8+ T lymphocytes are in a non-responsive or anergic (Reignat et al., 2002), suggesting that neutralising anti-HBs antibodies alone cannot eliminate infection. This observation was supported by an earlier vaccination study with recombinant HBsAg (Hepacare®), which contains Pre-S1, Pre-S2 and S proteins (Jung et al., 2002). The study showed that vaccination with HBsAg alone did not induce specific CD8+ T-cell responses using ELISPOT-assay and was accompanied by the production of Th2 cytokines (Jung et al., 2002). Another recent vaccine trial also reported that a combination of Lamivudine treatment, with a vaccine expressing HBsAg did not demonstrate superior clinical efficacy in CHB patients as compared to Lamivudine therapy alone (Vandepapelière et al., 2007). Similar observation was also reported in other studies that showed vaccination with HBsAg induced transient specific immune responses but was not translated into therapeutic efficacy, failing to control HBV infection in patients with
Previously, a study with randomised, non-blinded trial of Lamivudine treatment in combination with “prime-boost” vaccination using DNA vaccine and MVA that express HBsAg also did not reduce viraemia and failed to control HBV infection (Cavenaugh et al., 2011). A recent nasal vaccine containing both recombinant HBsAg and HBcAg used in a phase I double-blind, placebo-controlled randomised clinical trial, was highly immunogenic in healthy male adults with no serological markers of immunity to HBV prior to vaccination (Betancourt et al., 2007).

In our current study in Chapter 6, it was interesting that robust anti-DHBs antibody production was observed in ducks treated with ETV plus “prime-boost” vaccines expressing both DHBV core and surface antigens (Group 6A). This suggests that DNA vaccines expressing DHBcAg could provide help for the anti-DHBs antibody production in ducks treated with ETV plus “prime-boost” vaccines expressing both DHBV core and surface antigens (Group 6A). In the woodchuck model, a DNA vaccine expressing WHcAg induced a strong Th2 response that also enhances the development of anti-WHV surface antibodies (Lu et al., 2005). Our current data corroborates the previous reports on HBcAg specific helper T lymphocytes have the unique ability to sustain anti-HBs antibody production (Milich et al., 1987; Lobaina et al., 2005; Akbar et al., 2012). However, it is acknowledged that only two out of the five Group 6A ducks showed higher anti-DHBs antibody responses compared to Group 6B and thus this observation may need further confirmation.

In a recent study, four DHBV-infected ducks were treated with Lamivudine (during weeks 1-8 p.i.) in combination with DNA vaccine expressing DHBV core alone (5 doses, 300 µg each dose) starting from week 6 p.i. (Thermet et al., 2008). After 10 months of follow-up, liver
DHBV DNA was detected in the liver without the presence of serum anti-DHBs antibodies, indicating the failure of immune responses to clear DHBV infection. In contrast, the data in Chapter 6 showed that DHBV core (Group 6C) alone is able to induce specific immune responses to control DHBV infection using our current ETV treatment in combination with “prime-boost” protocol. It was of interest to note that detectable levels of anti-DHBs antibody production were not observed in these ducks (Group 6C) by ELISA. The data from this current study suggests that DHBV core alone was not able to induce protective humoral response but induced effective CMI responses that allowed the elimination of DHBV-infected hepatocytes and prevented the development of widespread DHBV-infection. Again, due to the limitation on duck specific reagents, specific CMI responses were not able to be tested in this study, which should provide a better understanding of the vaccine-induced immune responses to DHBV infection and its role during DHBV clearance.

In summary, it is believed that by incorporating multiple HBV antigens into potential therapeutic vaccines, the product will more closely imitate natural HBV infection and will be more likely to increase T lymphocyte responsiveness and subsequently break the immune tolerance induced by HBV. Thus, the role of HBcAg should be further assessed in patients with CHB and HBcAg should be an integral part of a therapeutic vaccine against chronic HBV infection.

7.5 Future directions

Currently, mAb to DHBV S antigen to detect the Australian strain DHBV are not available in our Laboratory. Thus, the mAb production techniques from Chapter 3 could be applied to generate antibodies specific to DHBV S using DHBsAg particles purified from DHBV-positive duck sera by cesium chloride density gradient centrifugation (Miller et al., 2004). MAb to the purified DHBV S could then be generated using similar procedures to those
described in Sections 2.3.2 and 2.3.3 and DHBV S specific antibodies could be screened for using ELISA and antibody specificity for the DHBV pre-S or S domains could be verified by Western Blot. In previous attempts to produce anti-DHBV S mAbs from mice immunised with purified DHBsAg particles, the majority of mAbs selected have specificity for the DHBV pre-S (Darren Miller, personal communication). As an alternative approach, anti-DHBV S mAb could be produced by immunising mice with DNA vaccines expressing DHBV S alone as described in Section 2.3.3. Whilst this approach has been shown to result in lower antibody responses in immunised mice, this should allow selection of hybridomas specific only to DHBV S, thus avoiding the immune dominance of the DHBV pre-S epitope.

The protective efficacy of DuCD40L expression constructs in combination with DNA vaccines was explored in Chapter 4 and Chapter 5. It is well documented that CD40L is a powerful immunological adjuvant that induces humoral and CMI responses (Garside et al., 1998; Aruffo et al., 1993; Daoussis et al., 2004; Fraser et al., 2007). In general, patients whose acute HBV infection resolves display strong multi-specific CD4+ and CD8+ T responses, with the secretion of Th1 antiviral cytokines and the production of neutralising anti-HBs antibodies (Bertoletti et al., 2006). In contrast, patients with CHB have functionally impaired, weak or even undetectable HBV-specific T cell immune responses (Rehermann et al., 1996). Based on these observations, therapeutic vaccination approaches that can enhance antiviral immune responses against HBV infection, particularly CD4+ and CD8+ T responses, should be a promising strategy for treating CHB.

In this regard, it would be particularly important to study a wide range of cytokines using RT-PCR. These include Th1 (e.g., IL-2, IL-12, IFN-γ) and Th2 (e.g., IL-4, IL-5, IL10) cytokines that involve in the activation of other immune cells, particularly T lymphocytes (Narayan et al., 2006; Puro and Schneider, 2007). By studying the expression profiles of these cytokines, this might indirectly help us to understand how DuCD40L expression
constructs affects T-cell responses. In addition, these cytokines can also be further explored as potential therapeutic tools for treatment of CHB.

Furthermore, CD40L on CD4+ T lymphocytes have been shown to license DC via CD40 to prime CTL responses (Bennett et al., 1998; Schoenberger et al., 1998; Smith et al., 2004). Immuno-staining of CD4+ and CD8+ T lymphocytes using anti-duck CD4 and CD8 monoclonal antibodies (Kothlow et al., 2005) could also be performed on duck liver sections to study how DuCD40L affects T lymphocyte infiltration in the liver following vaccination. If overall higher levels of CD8+ T lymphocytes were found in ducks that were resolving DHBV infection than ducks with persistent DHBV infection, this would suggest the importance of DuCD40L in activation of CD8+ T lymphocytes in the clearance of DHBV infection.

In this current post-exposure vaccination study (Chapter 6), the success of our ETV treatment in combination with “prime-boost” vaccination protocol suggested this vaccination protocol has potential as a therapeutic HBV vaccine. However, a recent study performed by our group showed that when this vaccination protocol was delayed for 5 days in 14-day-old ducks that infected with 5x10⁸ DHBV genomes, a dose known to cause persistent infection in ducks of this age (Foster et al., 2005), did not successfully clear DHBV-infected hepatocytes and ultimately the DHBV infection rebounded in all treated ducks with serum DHBsAg levels comparable to the vector vaccinated ducks (Dr Feng Feng, personal communication).

To further improve the immunogenicity of our ETV treatment in combination with “prime-boost” vaccination protocol, methodologies to administer DuCD40L and the DHBV antigens (e.g., S protein, pre-S/S protein or the core protein) as fusion proteins are worth assessing. Future work will also have to further determine the effectiveness of DuCD40L as an adjuvant to DNA vaccines by delaying the treatment of DHBV infection to allow higher initial levels
of infected hepatocytes at the onset of treatment. This will allow us to determine if virus spread prior to the DuCD40L plus DHBV DNA vaccination reduces the robustness of the immune responses. Another potential approach is to clone the DuCD40L gene into the FPV vaccine strain FPV-M3 for use in our “prime-boost” studies in ducks infected with DHBV. In a recent study, recombinant poxvirus MVA and a highly attenuated vaccinia virus strain, NYVAC, in heterologous DNA “prime” and poxvirus “boost” regimens, combined with multimeric soluble CD40L have been shown to be capable of inducing broad HIV-specific CMI in a mouse model (Gomez et al. 2009). This finding provides evidence for the adjuvant effect of CD40L on induction of specific immune responses to targeted antigens triggered by DNA “prime” and poxvirus “boost” regimens.

The use of another HBV antigen such as HBV polymerase, or with novel amphipathic DNA polymers (APDPs) (Vaillant et al., 2006) might further increase the efficacy of our therapeutic vaccination protocol. HBV polymerase represents the largest HBV protein and HBV polymerase specific CD4+ T cell responses have been demonstrated more frequently in patients with acute HBV infection or in patients after recovery from chronic HBV infection (Mizukoshi et al., 2004). In addition, transient increases in HBV polymerase specific CD4+ T cell responses with reduced levels of virus replication were observed in patients with CHB (Mizukoshi et al., 2004). Furthermore, HBV polymerase specific CD8+ T lymphocytes have been detected in patients with acute HBV infection (Rehermann et al., 1995). These results suggest HBV polymerase epitopes could be a valuable component of therapeutic vaccines. The HBV polymerase should be cloned into the same plasmid vector (pcDNA3) used to express DHBV antigens in this study and incorporated in our vaccination protocol to evaluate its therapeutic efficacy in the duck model. However, significantly lower numbers of polymerase antigens expressed on infected hepatocytes compared to other HBV antigens might limit the antiviral activities of HBV polymerase-specific T lymphocytes (Kakimi et al., 2002; Mizukoshi et al., 2004).
On the other hand, APDPs were developed by REPLICor Incorporation and have been shown to inhibit HIV-1-mediated membrane fusion and HIV-1 replication in a size dependent but sequence independent manner (Vaillant et al., 2006). The amphipathic nature of APDPs play a major role in their antiviral activities and it was proposed that the fusion process of HBV with the cell membrane can be blocked by APDPs (Noordeen Ph.D. thesis, 2009). Recently, a study performed by our group using APDP 2055 showed that it had excellent therapeutic efficacy against persistent DHBV infection. In the study, ducks with persistent DHBV infection (>95% of DHBV-infected hepatocytes) were treated with daily APDP 2055 (10 mg/kg) for 28 days. These ducks were monitored for 113 days after stopping treatment. The results showed that 56% of ducks treated with APDP 2055 were protected from rebound of DHBV infection with the development of anti-DHBs antibody responses, suggesting the resolution of DHBV infection (Noordeen Ph.D. thesis, 2009; Noordeen et al., 2013). This APDP 2055 approach should be explored further with our “prime-boost” vaccination protocol in the duck model to evaluate the effectiveness of APDP in the treatment of persistent DHBV infection.

Altogether, these finding suggest that virus suppression, stimulation of antiviral immune responses by DNA vaccines, viral vector vaccines, immunological adjuvant constructs and some novel approach such as APDPs have to combine together to counteract the negative effects of the immunosuppressive environment induced by HBV. However, prior to the development of therapeutic vaccines, several factors such as the duration of infection, immune tolerance status of the patients, the dosage and the antigen chosen and duration of therapy must be taken into account.
7.6 How these studies relate to HBV infection

The past decades have seen an explosion in our understanding of HBV infection and the important of host and virus responses in resolution of HBV infection (acute HBV infection) (Bertoletti and Gehring, 2006; Michel and Tiollais, 2010; Damme et al., 2013). As described previously in Chapter 1, patients whose HBV infection is resolving displays strong and functional humoral and CMI responses against HBV antigen, resulting in seroconversion of HBsAg to anti-HBs antibodies and/or HBeAg to anti-HBe antibodies. This seroconversion is followed by sustained immune control and thus our current vaccination strategies aim to mimic this natural seroconversion phenomenon.

The DHBV model has been pivotal in our current studies to improve our understanding of our novel therapeutic vaccination strategies against virus infection as described in Chapter 4, 5 and 6. These vaccination strategies could be explored as potential therapeutic tools to help clear CHB. Currently, CD40L expression construct (Chapter 4 and 5), DNA vaccines (Chapter 4 and 5), ETV (Chapter 6), and rFPV-vaccines (Chapter 6) have all been approved, albeit individually, for clinical use in humans without any observed adverse effects (Kent et al., 1998; Chang et al., 2006; Suzuki et al., 2008; Malmstrom et al., 2010; Cavenaugh et al., 2011; Keefer et al., 2011).

ETV is a powerful inhibitor of HBV replication, with the resistance rate to ETV treatment as low as 1.2% within 5 to 6 years in NA-naïve patients (Tenney et al., 2009a, b). To date, data of combination treatment of ETV and DNA vaccines in patients with CHB is not available. Notably, the development of quasispecies complexity and diversity during CHB affects the outcome of antiviral therapy (Liu et al., 2011; Desmond et al., 2012; Tong et al., 2013). Incidents of renal toxicity, lactic acidosis, neuropathy and myositis have also been reported associated with long-term ETV treatment (Woo et al., 2010). Thus, monitoring the HBV
quasispecies complexity, future drug-resistance, drug toxicity and associated adverse effects are important in predicting long-term antiviral ETV treatment outcomes.

The use of ETV in combination with “prime-boost” immunotherapy described in Chapter 6 provides a promising protocol to treat CHB. Previous non-related studies have shown the ability of this combination therapy to induce specific CD4+ and CD8+ T-cell responses (Kent et al., 1998; Walther et al., 2006; Rerks-Ngarm et al., 2009; Keefer et al., 2011), which is regarded to be an important component of immunotherapy for CHB. Previous reports also suggest that doses of DNA and rFPV-vaccines need to be optimised in order to be immunogenic in humans (Coupar et al., 2006; De Rose et al., 2006). Given that CD40-CD40L interactions are important for initiating humoral and CMI responses, it would not be surprising that co-expression of CD40L can maximise the immunological efficacy of our novel vaccination approaches against CHB in human.

Recent publication showed that the rFPV-vaccine requires help from T-lymphocytes, particularly the CD40-CD40L interactions, to induce efficient CMI responses (Diener et al., 2008). In this study, the addition of anti-CD40 mAbs significantly improved the rFPV-vaccine response, particularly the antigen-specific CD8+ T-lymphocyte memory formation. Likewise, another supporting study showed that complete tumour regression which relies on the activity of CD8+ T lymphocytes, was observed in B-cell lymphoma-bearing mice treated with chemotherapy and co-administered with CD40L-expressing rFPV (Liu et al., 2007). Taken together, it is logical to incorporate a CD40L expression by plasmid or viral vectors in future therapeutic protocols to enhance the vaccine-induced immune responses in patients with CHB.

Other concerns need to taken into consideration before the combination therapy could be tested in patients with CHB. For instance, safety concerns need to be evaluated if the vaccine-
induced immune attack on HBV-infected hepatocytes can result in acute liver failure and if there is enough hepatocyte proliferation to cover the liver cell death. It is known that CHB can be viewed in four stages: immune tolerance, immune clearance, immune control and reactivation (Yim and Lok, 2006; Kwon and Lok, 2011). Given that ongoing liver damage in patients with CHB can lead to the development of severe, decompensated liver diseases, ascites, fibrosis, cirrhosis and HCC depending on the stage of CHB (Jilbert et al., 2008), infection history, stage of CHB and liver inflammation need to be confirmed before treatment, to maximise the therapeutic efficacy with minimal clinical complications.

Lastly, to design new vaccination strategies for treatment of CHB, better understandings of the mechanisms involved in maintaining the persistence of HBV infection and in HBV clearance are needed. A clearer mechanistic understanding will enable the improvement of our vaccine efficacy either through genetic modification of the vector or its adjuvant component.

7.7 Concluding remarks

From studies conducted during this Ph.D., specific polyclonal and mAbs for the detection of DHBcAg were generated. The protocols for immuno-staining, Western Blotting and IMF detection of DHBcAg using these anti-DHBcAg-specific antibodies were also developed and optimised. The methods developed in this thesis are being applied in several other studies in our research group. The protective efficacy of DuCD40L expression construct on DNA vaccine was also extensively studied to increase the immunogenicity of DNA vaccination protocol. The efficacy of ETV treatment in combination with “prime-boost” vaccination expressing either DHBV surface or core as a single vaccine antigen was tested. The ultimate aim of our research is contribute new information to provide new directions for future therapeutic vaccination strategies for chronic HBV infection.
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