



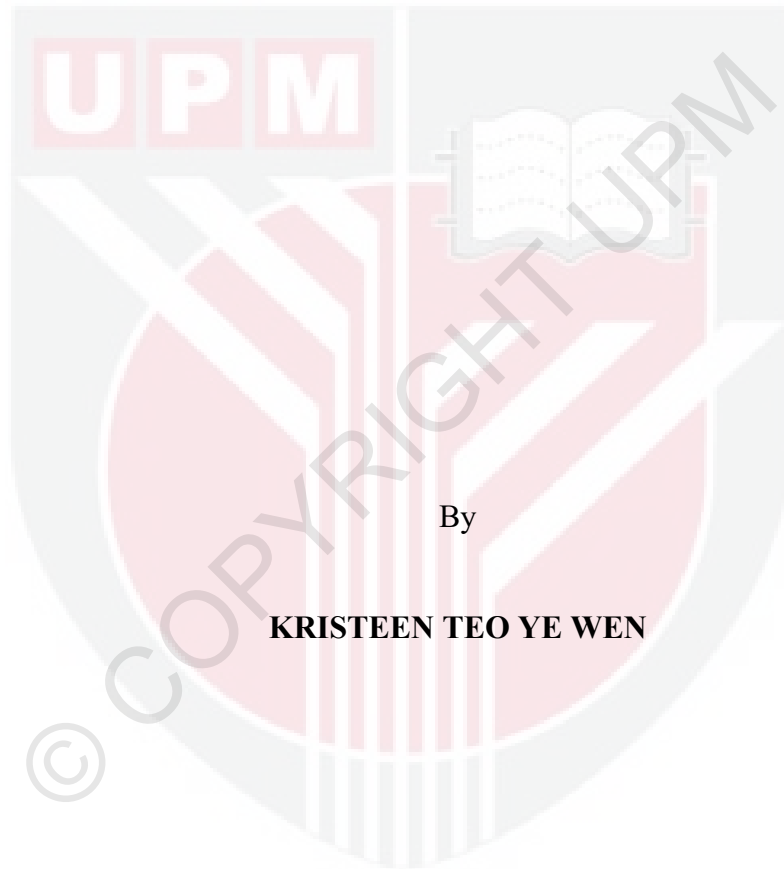
***IMMUNOREGULATION OF CHICKENS' B LYMPHOCYTES AND
THREE-DIMENSIONAL LYMPHOID TISSUE CULTURE
INFECTED WITH NEWCASTLE DISEASE VIRUS AND
INFECTIOUS BURSAL DISEASE VIRUS***

KRISTEEN TEO YE WEN

IB 2014 20



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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of the Requirements for the Degree of Master of Science**

February 2014

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

**IMMUNOREGULATION OF CHICKENS' B LYMPHOCYTES AND
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INFECTED WITH NEWCASTLE DISEASE VIRUS AND
INFECTIOUS BURSAL DISEASE VIRUS**

By

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February 2014

Chairman : Associate Professor Noorjahan Banu Mohamed Alitheen, PhD
Institute : Bioscience

Among the common poultry disease worldwide, Newcastle Disease (ND) and Infectious Bursal Disease (IBD) are contagious and pose a major threat in devastating the poultry industry. Numerous studies were carried out to evaluate host response of avian lymphocytes against Newcastle Disease Virus (NDV) and Infectious Bursal Disease Virus (IBDV) infection, but none has reported on the effect of these highly pathogenic viruses on pure B cells population. As B cell lineage lymphocytes are responsible for the production of antibodies, which play a role in preventing viral infection, this study investigated the responses of enriched B lymphocytes following infection of highly pathogenic NDV and very virulent IBDV strains. Cell viability and proliferation rate of *in-vitro* cultured B lymphocytes were assessed upon NDV and IBDV infection and results showed that other than the virus infection dosage, time course infection of the virus also affected the viability and inhibited the proliferation of B lymphocytes population in the culture. In the *in-vivo* study, chickens' spleen and bursa of Fabricius were investigated on their cell population changes and oxidative stress in relationship with the B lymphocytes response towards the infection of different genotypes of NDV and IBDV. NDV caused increment of macrophage in the organ which led to the elevation of nitric oxide content and NDV genotype VIII induced greater chronic impairment in chickens' spleen and bursa B cells with lower viral load detected compared to the infection by NDV Genotype VII. *In-vivo* study with IBDV infection revealed that the virus caused more severe damage in chicken bursa of Fabricius compared to the spleen. Further details showed that the depletion of B lymphocytes in chicken spleen is more relevant to the oxidative stress caused by the virus infection rather than the amount of virus residue in the cells. Meanwhile, the cell death event in B lymphocytes from bursa was in an increasing manner considerably with time of infection and viral load detected in the cells.

The second part of the study demonstrated the establishment of an *in-vitro* culture of chicken lymphoid tissue, simulating chicken embryonic bursa of Fabricius to study the interaction of chicken embryonic B cells upon infection with NDV and IBDV. Following infection with the viruses, cell population changes, viability, apoptosis and viral load were investigated. Results showed that IBDV caused drastic depletion in the matured (IgM+) and immature B cell (Bu-1a) populations while NDV infection induced the production of IgM+ cells, maybe as an effort to combat the virus infection. Cell death by apoptosis was assayed and the result showed that the *in-vitro* culture of chicken lymphoid tissue is susceptible to NDV and IBDV infection as higher titer of virus infection caused higher frequency of apoptosis as well as higher amount of viral load detected in the culture. The findings showed that the *in-vitro* model of chicken lymphoid tissue can be used to study the virus and host cells interaction. Moreover, the phenotypic and cell viability changes in the established mini organ upon virus infection are similar to previous reports by other researchers in their *in-vitro* and *in-vivo* approaches. In conclusion, B lymphocytes from chicken spleen and bursa of Fabricius at different age reacted unlike when infected with different strains of pathogenic NDV and IBDV. The depletion of the B lymphocytes population may be caused by population changes, oxidative stress or amount of virus resides in the cells.

Abstrak tesis yang dikemukakan kepada Senate Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

**RESPON REGULASI-IMUN B LIMFOSIT DAN TISU LIMFOID AYAM
TIGA DIMENSI TERHADAP JANGKITAN VIRUS NEWCASTLE DAN
BURSA BERJANGKIT**

Oleh

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Antara penyakit ayam yang lazim berlaku di seluruh dunia adalah penyakit Newcastle (ND) dan penyakit berjangkit Bursal (IBD) yang menjadi ancaman utama dalam industri penternakan ayam. Banyak kajian telah dijalankan untuk menilai respons limfosit burung terhadap Virus Penyakit Newcastle (NDV) dan Virus Penyakit Jangkitan Bursa (IBDV), tetapi tiada laporan mengenai kesan terhadap populasi limfosit B tulen. Oleh kerana limfosit B bertanggungjawab untuk penghasilan antibodi yang memainkan peranan penting dalam mencegah jangkitan virus, kajian ini bertujuan untuk mengkaji respon limfosit B yang telah diperkaya selepas jangkitan NDV dan IBDV. Akibat jangkitan NDV dan IBDV, kebolehhidupan dan kadar proliferasi limfosit B telah dikaji secara *in-vitro* dan keputusan menunjukkan bahawa selain daripada dos jangkitan, masa jangkitan turut menjejaskan kebolehhidupan and menghalang proliferasi limfosit B. Dalam kajian *in-vivo*, siasatan terhadap perubahan populasi sel dan kesan tekanan oksidatif kepada limpa dan bursa Fabricius ke atas respon limfosit B telah dijalankan selepas jangkitan NDV dan IBDV yang berlainan genotip. Keputusan telah menunjukkan bahawa NDV menyebabkan peningkatan frekuensi makrofaj dalam kedua-dua organ dan seterusnya menyebabkan peningkatan kandungan oksida nitrik. NDV genotip VIII menyebabkan kemerosotan kronik yang lebih teruk ke atas sel limpa ayam dan bursa dengan kuantiti virus yang lebih rendah berbanding dengan jangkitan oleh NDV genotip VII. Kajian *in vivo* dengan jangkitan IBDV mendedahkan bahawa virus tersebut menyebabkan kerosakan yang lebih teruk pada bursa ayam berbanding limpa. Maklumat selanjutnya menunjukkan bahawa penyusutan limfosit B dalam limpa ayam adalah kerana tekanan oksidatif yang disebabkan oleh jangkitan virus. Sementara itu, kematian sel limfosit B dalam Bursa didapati meningkat sejajar dengan masa jangkitan dan peningkatan jumlah virus yang dikesan di dalam sel.

Bahagian kedua kajian ini mendemostrasikan pembentukan kultur tisu limfoid ayam tiga dimensi secara *in vitro*, yang mensimulasi bursa Fabricius embrio ayam untuk mengkaji interaksi antara sel antibodi embrio terhadap jangkitan NDV dan IBDV. Selepas jangkitan virus, perubahan populasi, kebolehidupan, apoptosis dan kuantiti virus telah dikaji. Keputusan menunjukkan IBDV menyebabkan penyusutan drastik dalam populasi antibodi IgM+ matang dan sel B kurang matang sementara itu jangkitan NDV pula mendorong penghasilan sel IgM+ yang mungkin disebabkan oleh tindak balas sel untuk mencegah jangkitan virus tersebut. Ujian telah dijalankan untuk melihat kematian sel secara apoptosis dan hasil ujian menunjukkan bahawa kultur *in vitro* tersebut boleh dijangkiti oleh IBDV dan NDV kerana kepekatan virus yang tinggi menyebabkan frekuensi apoptosis dan kuantiti virus di dalam kultur tersebut meningkat. Penemuan ini menunjukkan bahawa model *in vitro* sel kultur limfoid ayam boleh digunakan untuk mengkaji interaksi antara virus dan sel perumah. Tambahan pula, keputusan perubahan populasi sel dan kebolehidupan sel di dalam organ mini semasa jangkitan virus adalah seiring dengan keputusan yang dilaporkan oleh penyelidik yang lain dalam pendekatan *in vitro* dan *in vivo* mereka. Kesimpulannya, sel B daripada limpa dan bursa ayam mempunyai tindak balas yang berbeza mengikut masa jangkitan apabila dijangkiti oleh IBDV dan NDV yang berbeza. Penyusutan populasi sel limfosit B mungkin disebabkan oleh perubahan populasi, tekanan oksidatif atau kuantiti virus yang menjangkiti sel.

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I certify that a Thesis Examination Committee has met on 4 September 2014 to conduct the final examination of Kristeen Teo Ye Wen on her thesis entitled "Immunoregulation of Chickens' B Lymphocytes and Three-Dimensional Lymphoid Tissue Culture Infected with Newcastle Disease Virus and Infectious Bursal Disease Virus" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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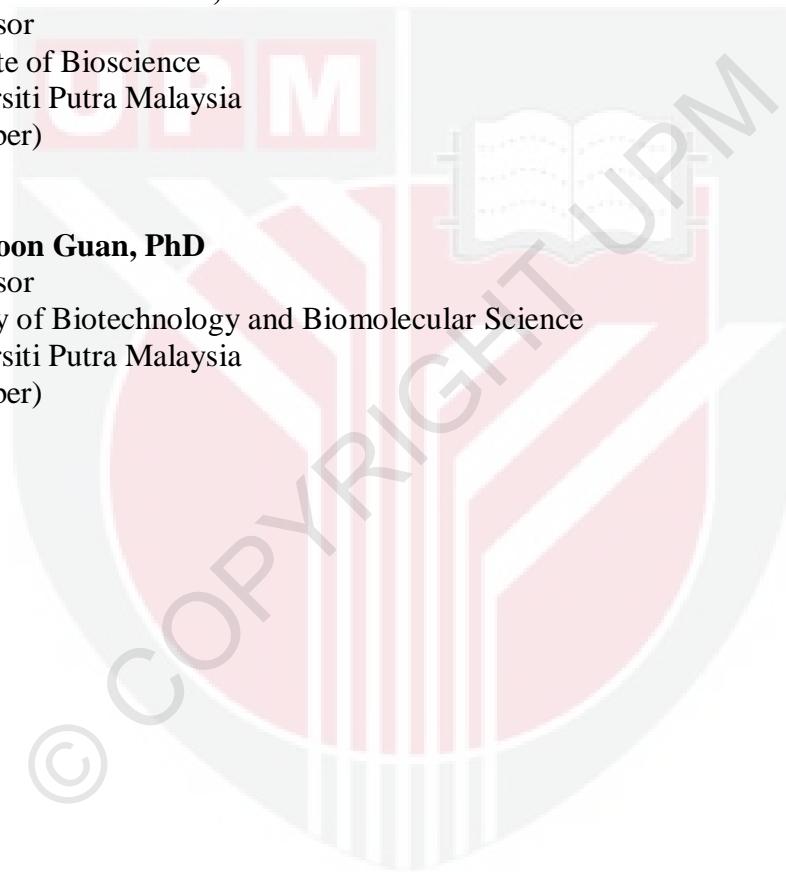
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TABLE OF CONTENTS

| | Page |
|---|-------------|
| ABSTRACT | i |
| ABSTRAK | iii |
| ACKNOWLEDGEMENTS | v |
| APPROVAL | vi |
| DECLARATION | viii |
| LIST OF TABLES | xiv |
| LIST OF FIGURES | xvi |
| LIST OF ABBREVIATIONS | xix |
| CHAPTER | |
| 1 INTRODUCTION | 1 |
| 2 LITERATURE REVIEW | 3 |
| 2.1 Avian Lymphoid Organ | 3 |
| 2.1.1 Bursa of Fabricius | 3 |
| 2.1.2 Spleen | 4 |
| 2.2 Avian Adaptive Immunity | 4 |
| 2.2.1 Humoral response of avian | 4 |
| 2.3 Newcastle Disease virus (NDV) | 5 |
| 2.3.1 Virulence of NDV | 6 |
| 2.3.2 Genome organization of NDV | 7 |
| 2.3.3 Infection cycle of NDV | 8 |
| 2.3.4 Diagnosis of NDV | 9 |
| 2.3.5 Avian host responses to NDV infection | 10 |
| 2.4 Infectious Bursal Disease Virus (IBDV) | 11 |
| 2.4.1 Virulence of IBDV | 11 |
| 2.4.2 Genome organization of IBDV | 11 |
| 2.4.3 Diagnosis of IBDV | 13 |
| 2.4.4 Avian host responses to IBDV infection | 14 |
| 2.5 Apoptosis | 15 |
| 2.5.1 Apoptotic strategies related to infection of virus | 15 |
| 2.6 <i>In-vitro</i> Three Dimensional Cell Culture | 15 |
| 2.7 Analytical Methods | 16 |
| 2.7.1 Cell Viability Study | 16 |
| 2.7.1.1 MTT assay | 16 |
| 2.7.1.2 AO/PI double staining assay | 16 |
| 2.7.1.3 Cell cycle analysis | 17 |
| 2.7.1.4 Annexin V assay | 17 |
| 2.7.2 Oxidative Stress Study | 17 |
| 2.7.2.1 Inflammatory response: nitric oxide measurement | 18 |
| 2.7.2.2 Lipid peroxidation :Malondialdehyde (MDA) detection | 18 |
| 2.8 Concluding Remarks | 18 |

| | | |
|----------|---|-----------|
| 3 | RESPONSES OF ENRICHED CHICKEN B LYMPHOCYTES POPULATION TOWARDS INFECTION OF DIFFERENT GENOTYPES OF VELOGENIC NEWCASTLE DISEASE VIRUS | 19 |
| 3.1 | Introduction | 19 |
| 3.2 | Materials and Methods | 20 |
| 3.2.1 | Reagents and chemicals | 20 |
| 3.2.2 | Virus strains | 20 |
| 3.2.2.1 | Haemagglutination test | 20 |
| 3.2.3 | In-vitro screening of chicken enriched B lymphocytes following NDV infection | 21 |
| 3.2.3.1 | Enrichment of B cell population | 21 |
| 3.2.3.2 | Inoculation of NDV | 21 |
| 3.2.3.3 | Trypan blue exclusion assay | 21 |
| 3.2.3.4 | MTT assay | 22 |
| 3.2.3.5 | BrdU assay | 22 |
| 3.2.4 | In-vivo response of enriched chicken B lymphocytes upon infection of different genotypes of N NDV | 22 |
| 3.2.4.1 | Experiment design | 22 |
| 3.2.4.2 | Immunophenotyping of SPF chicken spleen and bursa of Fabricius | 23 |
| 3.2.4.3 | Nitric Oxide Assay | 23 |
| 3.2.4.4 | Enrichment of B cell Population | 23 |
| 3.2.4.5 | MTT assay | 23 |
| 3.2.4.6 | AO/PI double staining assay | 23 |
| 3.2.4.7 | DNA cell cycle analysis | 24 |
| 3.2.4.8 | Annexin V Apoptosis Assay | 24 |
| 3.2.4.9 | Quantification of viral load | 24 |
| 3.3 | Statistical Analysis | 25 |
| 3.4 | Results | 25 |
| 3.4.1 | <i>In vitro</i> screening of Enriched B Lymphocytes upon Infection of NDV | 25 |
| 3.4.1.1 | Cell Viability and Proliferation Assessment | 25 |
| 3.4.2 | <i>In vivo</i> Response of Enriched Chicken B Lymphocytes Upon Infection of NDV | 27 |
| 3.4.2.1 | Immunophenotyping | 27 |
| 3.4.2.2 | Nitric oxide Detection | 32 |
| 3.4.2.3 | Cell Viability Assay (Trypan Blue and MTT) | 33 |
| 3.4.2.4 | Acridine Orange/ Propidium Iodide (AO/PI) Assay | 35 |
| 3.4.2.5 | DNA Cell Cycle Analysis | 36 |
| 3.4.2.6 | AnnexinV Study | 38 |
| 3.4.2.7 | Real-Time PCR Quantification of Viral Load | 40 |
| 3.5 | Discussion | 41 |

| | | |
|----------|--|-----------|
| 4 | RESPONSES OF ENRICHED CHICKEN B LYMPHOCYTES POPULATION TOWARDS INFECTION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS | 45 |
| 4.1 | Introduction | 45 |
| 4.2 | Materials and Methods | 45 |
| 4.2.1 | Reagents and chemicals | 45 |
| 4.2.2 | Virus Strain | 46 |
| 4.2.3 | In-vitro screening of enriched chicken B lymphocytes following IBDV infection | 46 |
| 4.2.3.1 | Enrichment of B cell population | 46 |
| 4.2.3.2 | Inoculation of IBDV | 46 |
| 4.2.3.3 | Trypan blue exclusion assay | 46 |
| 4.2.3.4 | MTT assay | 47 |
| 4.2.3.5 | BrdU assay | 47 |
| 4.2.4 | <i>In-vivo</i> response of enriched chicken B lymphocytes upon infection of IBDV | 47 |
| 4.2.4.1 | Experimental design | 47 |
| 4.2.4.2 | Immunophenotyping of chicken spleen and bursa of Fabricus | 47 |
| 4.2.4.3 | Nitric oxide detection | 47 |
| 4.2.4.4 | MDA assay | 47 |
| 4.2.4.5 | Enrichment of B cell population | 48 |
| 4.2.4.6 | MTT assay | 48 |
| 4.2.4.7 | AO/PI assay | 48 |
| 4.2.4.8 | DNA cell cycle analysis | 48 |
| 4.2.4.9 | Annexin V Assay | 48 |
| 4.2.4.10 | Quantification of viral Load | 48 |
| 4.3 | Statistical Analysis | 49 |
| 4.4 | Results | 50 |
| 4.4.1 | <i>In vitro</i> screening of Chicken Pure B Lymphocytes Viability Upon Infection of IBDV | 50 |
| 4.4.1.1 | Cell Viability and Proliferation Assessment of in vitro Cultured B Lymphocytes Upon IBDV Infection | 50 |
| 4.4.2 | <i>In vivo</i> Response of Chicken Pure B Lymphocytes Upon Infection of IBDV | 52 |
| 4.4.2.1 | Immunophenotyping | 52 |
| 4.4.2.2 | Lipid peroxidation detection: MDA Assay | 54 |
| 4.4.2.3 | Nitric oxide content | 55 |
| 4.4.2.4 | Cell Viability Assessment upon IBDV Infection | 56 |
| 4.4.2.5 | Apoptosis Study upon IBDV Infection | 58 |
| 4.4.2.6 | DNA Cell Cycle Analysis | 60 |
| 4.4.2.7 | Annexin V Apoptosis Study | 61 |
| 4.4.2.8 | Real Time Quantification of IBDV Viral Load | 62 |
| 4.5 | Discussion | 64 |

| | | |
|----------|---|------------|
| 5 | RESPONSE OF IN-VITRO 3D CHICKEN LYMPHOID TISSUES AGAINST INFECTION OF HIGHLY PATHOGENIC NDV AND VERY VIRULENT IBDV | 67 |
| 5.1 | Introduction | 67 |
| 5.2 | Materials and Methods | 67 |
| 5.2.1 | <i>In-vitro</i> 3D chicken lymphoid tissues culture | 67 |
| 5.2.2 | CFSE cell trace assay | 68 |
| 5.2.3 | Inoculation of viruses | 68 |
| 5.2.4 | Cell viability assay | 68 |
| 5.2.5 | Flow cytometry detection of IgM+ and Bu-1a+ cells | 69 |
| 5.2.6 | DNA cell cycle analysis | 69 |
| 5.2.7 | Annexin V Study | 69 |
| 5.2.8 | Quantification of viral load | 69 |
| 5.3 | Statistical Analysis | 69 |
| 5.4 | Results | 70 |
| 5.4.1 | Optimization of the Establishment of <i>In vitro</i> 3D Chicken Lymphoid Tissue | 70 |
| 5.4.2 | Morphological Appearance of <i>In vitro</i> 3D Chicken Lymphoid Tissue | 72 |
| 5.4.3 | CFSE Cell Trace Assay | 73 |
| 5.4.4 | Cell Viability Assay | 75 |
| 5.4.5 | B Cell Surface Phenotyping Following Infection of NDV and IBDV | 76 |
| 5.4.6 | Cell Cycle Analysis | 77 |
| 5.4.7 | Annexin V Study | 78 |
| 5.4.8 | Real time Quantification of Viral Load in Mini Organ and Migrating Cells | 79 |
| 5.5 | Discussion | 80 |
| 6 | GENERAL DISCUSSION AND CONCLUSION | 83 |
| 6.1 | General Discussion | 83 |
| 6.2 | Conclusion and Future Recommendations | 85 |
| | REFERENCES | 87 |
| | APPENDICES | 105 |
| | BIODATA OF STUDENT | 111 |
| | LIST OF PUBLICATIONS | 112 |

LIST OF TABLES

| Table | Page |
|---|-------------|
| 2.1 The symptoms of NDV infection | 6 |
| 2.2 Differences of amino acid motif between NDV strains | 7 |
| 2.3 Description and role of different IBDV viral protein | 13 |
| 3.1 Viable cell counts of enriched B lymphocytes population after infection of NDV AF2240 at different time point using trypan blue | 26 |
| 3.2 Viable cell counts of enriched B lymphocytes population after infection of NDV AF2240 and IBS002 at different time point using trypan blue | 34 |
| 3.3 AO/PI Assay of Enriched B lymphocytes infected with NDV AF2240 | 36 |
| 3.4 FACS analysis of Annexin V-FITC binding of B lymphocytes in chicken spleen and bursa of 3-week-old chicken infected with NDV AF2240 and IBS002 at day 1, 3 and 4 post infection | 39 |
| 3.5 Taqman real time PCR result of detection of NDV AF2240 virus in the IgM+ cell population isolated from chicken spleen and bursa | 40 |
| 3.6 Taqman real time PCR result of detection of NDV IBS002 virus in the IgM+ cell population isolated from chicken spleen and bursa | 41 |
| 4.1 Thermal cycling protocol of SYBR Green based real time PCR in detecting IBDV viral load | 49 |
| 4.2 Viable cell counts of enriched B lymphocytes population after infection of IBDV UPM0081 at different time point using trypan blue | 50 |
| 4.3 Viable cell counts of enriched B lymphocytes population after infection of IBDV UPM0081 at different time point using trypan blue (in-vivo) | 57 |
| 4.4 AO/PI Assay of enriched B lymphocytes infected with IBDV UPM0081 | 59 |
| 4.5 SYBR Green real time PCR result of detection of IBDV UPM0081 virus in the cell populations isolated from chicken bursa | 63 |

| | | |
|-----|--|----|
| 4.6 | SYBR Green real time PCR result of detection of IBDV UPM0081 virus in the cell populations isolated from chicken spleen | 64 |
| 5.1 | The age of chick embryos (days) and the number of successful replicates per number of attempts | 70 |
| 5.2 | Double staining immunophenotyping of IgM+ and Bu-1a+ subpopulations in the mini organ and migrating cells following infection with NDV AF2240 and IBDV UPM0081 | 76 |
| 5.3 | Annexin V staining in the mini organ and migrating cells following infection with NDV AF2240 and UPM0081 | 78 |
| 5.4 | Real time PCR result of detection of NDV AF2240 virus in the mini organ and migrating cells following virus infection | 79 |
| 5.5 | Real time PCR result of detection of NDV AF2240 virus in the mini organ and migrating cells following virus infection | 80 |
| 6.1 | Summary of in vivo study upon NDV and IBDV infections | 84 |

LIST OF FIGURES

| Figure | | Page |
|---------------|---|-------------|
| 2.1 | Schematic representation of NDV virion structure | 8 |
| 2.2 | Genome organization and proteins of Infectious bursal disease virus | 12 |
| 3.1 | MTT cell viability assay of enriched B lymphocytes upon infection with different titer of NDV AF2240 at different timepoints | 26 |
| 3.2 | BrdU cell proliferation assay of enriched B lymphocytes upon infection with different titer of NDV AF2240 at different timepoints | 27 |
| 3.3 | The percentage of CD4+ cells (I), CD8+ cells (II), IgM+ cells (III) and macrophages (IV) in the bursa of 3-week-old chickens infected with AF2240 strains of NDV at 1, 3 and 4 day post infections | 28 |
| 3.4 | The percentage of CD4+ cells (I), CD8+ cells (II), IgM+ cells (III) and macrophages (IV) in the bursa of 3-week-old chickens infected with IBS002 strains of NDV at 1, 3 and 4 day post infections | 29 |
| 3.5 | The percentage of CD4+ cells (I), CD8+ cells (II), IgM+ cells (III) and macrophages (IV) in the spleen of 3-week-old chickens infected with AF2240 strains of NDV at 1, 3 and 4 day post infections | 30 |
| 3.6 | The percentage of CD4+ cells (I), CD8+ cells (II), IgM+ cells (III) and macrophages (IV) in the spleen of 3-week-old chickens infected with IBS002 strains of NDV at 1, 3 and 4 day post infections | 31 |
| 3.7 | The nitric oxide content in the spleen of 3-week-old chicken infected with NDVAF2240 (blue) and IBS002 (red) at day 1, 3 and 4 post infection | 32 |
| 3.8 | The nitric oxide content in the bursa of 3-week-old chicken infected with NDVAF2240 (blue) and IBS002 (red) at day 1, 3 and 4 post infection | 33 |
| 3.9 | Cell viability of B lymphocytes in the spleen of 3-week-old chicken infected with NDVAF2240 (blue) and IBS002 (red) at day 1, 3 and 4 post infection | 34 |

| | | |
|------|--|----|
| 3.10 | Cell viability of B lymphocytes in the bursa of 3-week-old chicken infected with NDVAF2240 (blue) and IBS002 (red) at day 1, 3 and 4 post infection | 35 |
| 3.11 | AO/PI assay of chicken enriched B lymphocytes infected with different genotype of NDV | 35 |
| 3.12 | Perturbations of cell cycle phases of B lymphocytes in the spleen of 3-week-old chicken infected with NDV AF2240 (I) and IBS002 (II) at day 1, 3 and 4 post infections | 37 |
| 3.13 | Perturbations of cell cycle phases of B lymphocytes in the bursa of 3-week-old chicken infected with NDV AF2240 (I) and IBS002 (II) at day 1, 3 and 4 post infections | 38 |
| 4.1 | Cell viability of enriched B lymphocytes upon infection with different concentration of IBDV UPM0081 at different time point | 51 |
| 4.2 | Cell proliferation rate of enriched B lymphocytes upon infection with different concentration of IBDV UPM0081 at different time point | 51 |
| 4.3 | The percentage of CD4+ cells (I), CD8+ cells (II), IgM+ cells (III) and macrophages (IV) in the spleen of 35 days-old chickens infected with IBDV UPM0081 at 2, 4 and 5 day post infection | 53 |
| 4.4 | The percentage of CD4+ cells (I), CD8+ cells (II), IgM+ cells (III) and macrophages (IV) in the bursa of 35 days-old chickens infected with IBDV UPM0081 at 2, 4 and 5 day post infection | 54 |
| 4.5 | The MDA content in the bursa and spleen of 35 days-old chicken infected with IBDV UPM0081 at 2, 4 and 5 post infection | 55 |
| 4.6 | The nitric oxide content in spleen (blue) and bursa (red) of 35 days-old chicken infected with IBDV UPM0081 at 2, 4 and 5 post infection | 56 |
| 4.7 | Cell viability of B lymphocytes in the bursa of 35 days-old chicken infected with IBDV UPM0081 at 2, 4 and 5 post infection | 57 |
| 4.8 | Cell viability of B lymphocytes in the spleen of 35 days-old chicken infected with IBDV UPM0081 at 2, 4 and 5 post infection | 58 |
| 4.9 | AO/PI of chicken enriched B lymphocytes from spleen infected with very virulent IBDV | 58 |
| 4.10 | AO/PI of chicken enriched B lymphocytes from bursa of Fabricius infected with very virulent IBDV | 59 |

| | | |
|------|---|----|
| 4.11 | Perturbations of cell cycle phases of B lymphocytes in the bursa of 35 days-old chicken infected with IBDV UPM0081 at 2, 4 and 5 post infection | 60 |
| 4.12 | Perturbations of cell cycle phases of B lymphocytes in the spleen of 35 days-old chicken infected with IBDV UPM0081 at 2, 4 and 5 post infection | 61 |
| 4.13 | FACS analysis of Annexin V-FITC binding of B lymphocytes in the bursa and spleen of 35 days-old chicken infected with IBDV UPM0081 at 2, 4 and 5 post infection | 62 |
| 5.1 | Different ages of chicken embryos | 71 |
| 5.2 | Morphological appearance of live culture macroscopically | 72 |
| 5.3 | Phase contrast microscopy of migrating cells from agglomerate | 73 |
| 5.4 | Proliferation of splenocytes analyzed by CFSE-labeling | 74 |
| 5.5 | Cell viability of in-vitro 3D chicken lymphoid tissue following infection with different titer of NDV AF2240 and IBDV UPM0081 after 48 hours | 75 |
| 5.6 | Cell cycle analysis of in-vitro 3D chicken lymphoid tissue following infection with different titer of NDV AF2240 and IBDV UPM0081 after 48 hours | 77 |

LIST OF ABBREVIATIONS

| | |
|-----------------|---------------------------------------|
| 2D | 2 Dimension |
| 3D | 3 Dimension |
| 7-AAD | 7-aminiactinomycin A |
| AGID | Agar gel immunodiffusion test |
| ANOVA | Analysis of Variance |
| AO/PI | Acridine Orange/ Propidium Iodide |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine Serum Albumin |
| CD | Cluster of differentiation |
| CDNA | Complementary deoxyribonucleic acid |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CO ₂ | Carbon dioxide |
| Cq | Quantification cycle |
| Ct | Threshold cycle |
| DDA | Dimethyl dioctadecyl ammonium bromide |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| F | Fusion protein |
| FACS | Fluorescence-activated cell sorter |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward Scatter |

| | |
|----------------------------------|--|
| HA | Haemagglutination |
| HI | Haemagglutination inhibition |
| HN | Hemagglutinin-neuraminidase |
| IBD | Infectious bursal disease |
| IBDV | Infectious bursal disease virus |
| IBS | Institute of Bioscience |
| ICPI | Intracerebral pathogenicity index |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| iNoS | Inducible nitric oxide synthase |
| M | Matrix protein |
| MDA | Malondialdehyde |
| MDA | Malondialdehyde |
| MgCl ₂ | Magnesium Chloride |
| MHC | Major histocompatibility complex |
| mm | Millimeter |
| mM | Millimolar |
| MOI | Multiplicity of infection |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MyCC | Malaysia Competition Commission |
| Na ₂ HPO ₄ | Disodium hydrogen phosphate |
| NaCl | Sodium chloride |
| NaHCO ₃ | Sodium bicarbonate |
| ND | Newcastle disease |
| NDV | Newcastle disease virus |

| | |
|--------|---|
| NO | Nitric Oxide |
| NP | Nucleoprotein |
| NTC | Non template control |
| OD | Optical density |
| OIE | Office International des Epizooties |
| ORF | Open reading frames |
| P | Phosphoprotein |
| PALS | Peri-arteriolar lymphoid sheath |
| PBS | Phosphate buffer saline |
| PCR | Polymerase Chain Reaction |
| PE | Phycoerythrin |
| PerCP | Peridinin chlorophyll |
| PI | Propidium iodide |
| PS | Phosphatidylserine |
| PWP | Peripheral white pulp |
| RNA | Ribonucleic acid |
| rpm | Rotation per minute |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| S.E.M | Standard Error Mean |
| SSC | Side Scatter |
| UPM | Universiti Putra Malaysia |
| vvIBDV | very virulent Infectious Bursal Disease Virus |

CHAPTER 1

INTRODUCTION

Poultry industry is considered one of the tremendously growing sectors in Malaysia. Among all livestock in this country, chicken meat is consumed the most due to cultural and religious reasons. According to the review of domestic broiler market report issued by Malaysia Competition Commission (MyCC), (2012), annual consumption of broiler meat have been increasing steadily from about 31 kg per capita in year 2000 to 37 kg per capita in year 2010. In term of supply side, the 546,000 day-old-chicks produced in 2010 was about 56% higher than the 364,000 chicks produced in 2000. The total quantity supplied of 524,000 broilers in 2010 was more than 63% higher than the 364,300 broilers supplied in 2000. To date, Malaysia has in continued with its self-sufficiency of poultry meat supply that was first achieved in 1990. Thus, this industry is regarded as one of the most important livestock sector in Malaysia. Due to its economic significance, it is crucial to monitor and have control measures taken to prevent losses and devastation caused by the poultry disease (Malaysia Competition Commission, 2012).

Among the major threats that cause heavy losses in the poultry industry throughout the world are the Newcastle Disease (ND) caused by Newcastle Disease Virus (NDV) and Infectious bursal Disease (IBD), which is an acute contagious virus disease of young birds caused by Infectious bursal Disease Virus (IBDV).

Newcastle Disease Virus has been classified into the order of *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Rubulavirus* (Alexander, 1988a). The virus primarily infects poultry and can be categorized into three pathotypes; 8which produces respiratory and nervous signs with moderate mortality and the viscerotropic or neurotropic velogenic strain which causes severe intestinal lesions or neurological disease resulting in high mortality (Alexander, 1988b; Aini, 1990).

The first outbreak of IBD was reported in Gumboro Delaware, USA (Cosgrove, 1962), caused by the infectious bursal disease virus (IBDV), an avian *Birnavirus*. The serotype 1 IBDV can be classified into different group based on their virulence: mild, classical virulent, very virulent and variant strains (Cao et al., 1998). The IBDV targets for cells of the bursa of Fabricius in chickens and infects the actively dividing and differentiating B cell lineage lymphocytes (Burkhardt and Muller., 1987). This virus causes severe acute disease on 3-6 weeks old birds with high mortality rate. However, when infecting chicks younger than 3 weeks old, the virus causes less acute disease (OIE, 2008).

Against the infection of virus, adaptive immunity consisting of antibodies and lymphocytes, known as the humoral and cell mediated responses are important (Abbas et al., 2007). The antibody response is crucial in preventing viral infections and may contribute to the resolution of the viral infection. Upon infection with virus, antibodies are produced against epitopes on viral protein and subsequently block the virus infection via virus neutralization (Klimpel, 1996). Antibodies work in various ways to neutralize viral infectivity such as interfering the virion binding to receptors, block uptake into cells, prevent uncoating of the genomes in endosomes, or cause aggregation of virus particles (Klimpel, 1996). The B cell lineage lymphocytes are the one responsible for the production of antibodies against the virus infection. It was reported that certain NDV strains caused decreased in B cell population (Russell et al., 1997) and resulted in a compromised immune response in the chickens. As for IBDV, the virus was also shown to target IgM bearing B lymphocytes (Ogawa et al., 1998). To date, no study has been reported on the effect of very virulent strains of NDV and IBDV on pure B cells population. Therefore in the initial part of the study, the interaction between enriched B cells population in chicken lymphoid tissues and the virus infection *in vitro* and *in vivo* was examined.

The second part of this study focused on the *in vitro* simulation of chicken lymphoid tissues against the infection of very virulent NDV and IBDV. A better defined geometry 3 dimensional (3D) culture is important to directly relate structure to function analysis. Today, there is increasing awareness of the drawbacks of 2D cell culture and the related effect on the value of the research being performed. Not surprisingly, scientists are shifting their focus to cells cultured in 3D. For example, human breast epithelial cells (Lee et al., 2007) and cardiac stem cells (Hosseinkhani et al., 2010) as well as analogues of mammalian B cell development 3D models (Henderson and Dorshkin, 1990; Nunzia et al., 2011) were developed for *in vitro* studies. However, comparable avian systems to mimic and evaluate B cell maturation are still lacking even though B cells were originally described in chick studies before extrapolation to mammals. Therefore in our study, we propose the establishment of an *in vitro* system of chicken lymphoid tissue which mimics the chicken bursa of Fabricius and offers us the advantage for manipulation under controlled condition and eliminates the need of sacrificing large amounts of animals. Such *in vitro* system should be able to support the replication of NDV and IBDV so that the interaction between the *in vitro* model and virus infection can be investigated.

Thus, the objectives of this study were:

- 1) To investigate and compare the response of chicken enriched B lymphocytes population upon infection with different strains of NDV and IBDV.
- 2) To establish an *in vitro* system of chicken lymphoid tissues mimicking chicken bursa of Fabricius upon infection of different strains of NDV and IBDV.

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LIST OF PUBLICATIONS

- Alitheen N.B., McClure S.J., Yeap S.K., Kristeen-Teo Y.W., Tan S.W., McCullagh P., 2012. Establishment of an *in vitro* system representing the chicken gut-associated lymphoid tissue. *PLOS ONE* 7(11): e49188
- Kristeen-Teo Y.W., Omar A.R., Yeap S.K., Tan S.W., Tan S.G., Alitheen N.B. Responses of enriched chicken B lymphocytes population towards infection of different genotypes of velogenic Newcastle disease virus. In Proceeding of WPSA (Malaysia Branch) and WVPA (Malaysia Branch) Scientific Conference 2013, 30 November – 1 December 2013, Faculty of Veterinary Medicine, Universiti Putra Malaysia
- Kristeen-Teo Y.W., Yeap S.K., Tan S.W., Kiarash R., Omar A.R., Tan S.G., Alitheen N.B., 2014. Genotype VII and VIII of velogenic Newcastle Disease virus induced differential macrophage infiltration and IFN- γ expression in bursa of infected SPF chicken. *Research in Veterinary Science*. Submitted
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