UNIVERSITI PUTRA MALAYSIA

IMMUNE SYSTEM REGULATION AND RESPONSE DURING INFECTIOUS BURSAL DISEASE VIRUS AND NEWCASTLE DISEASE VIRUS INFECTIONS IN CHICKENS

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IB 2013 31

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By

MEHDI RASOLI PIROZYAN

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

June 2013

Dedicated to:

My Parents, without whom I could never have come so far

&

My Beloved Sisters, who have loved me endlessly

&

Whoever has provided me with care and compassion throughout my life.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairperson: Professor Abdul Rahman Omar, PhD

Faculty: Institute of Bioscience

Infectious bursal disease (IBD) is caused by IBD virus (IBDV), a highly infectious lymphotropic virus that induces cytocidal effect on B lymphocytes of bursa Fabricius. Hence, the disease has been considered to have the most impact due to its immunosuppressive effects in young birds. However, the effects of the virus on non B lymphocytes functions and secretion of cytokines and chemokines are poorly characterized. On the other hand, Newcastle disease virus (NDV) is a highly infectious virus, which contributed to the major causes of economic losses in poultry industry. The virus can be different into several genotypes, however, velogenic NDV are of genotypes V, VI, VII, VIII and X. Hence, understanding IBDV and NDV immunoregulation on the host immune system will provide valuable information to define the immunopathology of the respective viruses.

In this study, immunophenotyping of lymphocytes and productions of cytokines and chemokines expression were analysed by using flow cytometry and GeXP/real-time PCR assays, respectively, in order to understand the roles of B, T cells and

macrophages during IBDV and NDV infections. Based on *in vitro* study, very virulent IBDV strain UPM0081 was detected in monocytes-macrophage cell line, HD11 cells as early as 6 hours post-infection. On the other hand, ConA-C1-Vick, a chicken $CD4^+$ and $CD8^+$ T cell line was not responding against IBDV infection *in vitro*. *In vitro* cytocidal effect of IBDV towards HD11 cell line showed evidence of apoptosis where 6% of cells undergo early apoptosis at 24 hours followed by 11% of cells undergo late apoptosis. Up-regulation of pro-inflammatory related cytokines/chemokines and other related genes such as CXCLi1, CXCLi2, CCL4, IL12α, IL-18, IL-1β, iNOS, TLR-3 and MHCI were detected in IBDV infected HD11 cell line. In the *in vivo* study, vvIBDV (UPM0081) was detected in both spleen and bursa as early as day 2 post-infection in specific-pathogen-free (SPF) chickens based on PCR detection. However, infiltration of Kul1+ macrophages population in spleen and bursa was different. Expressions of cytokines, chemokines and other immune-related genes in both spleen and bursa were compared in this study to understand the pathogenesis of vvIBDV infection. IL-10, an anti-inflammatory cytokine that commonly expressed by macrophage, was down-regulated in HD11 cells but no significant changes were detected in the bursa and spleen of the infected chicken throughout the study. Unlike constant increase of IL-6, IL-12α, and iNOS in bursa, highest level of IL-6 and IL-12α were found in spleen at day 2 days post-infections while iNOS was recorded with highest expression in spleen at day 3 post-infection. Added to this, IL-8 (CXCLi2) and IL-18 recorded the highest level of expression in day 3 and day 2 post-infection, respectively, in both bursa and spleen.

In the case of NDV, immunoregulation of velogenic NDV genotype VII (IBS002) and VIII (AF2240) on chicken macrophages, B and T lymphocytes during the acute stage of the respective virus infection in SPF chickens was characterized. Both NDV

genotypes induced drastic reduction of $CD4^+$ and $CD8^+$ T lymphocyte and associated with infiltration of macrophage in spleen at day 3 post-infection. The depletion of the T lymphocytes is probably through the process of apoptosis since 37% and 39% of ConA-C1-Vick cells undergo apoptosis at 24 and 48 hours post-infection, respectively. In addition, gene expression profiles showed an up-regulation of CCL4, CXCLi1, CXCLi2, IFN-γ, IL12α, IL-18, IL-1β, IL-6, iNOS, TLR-7, MHCI, IL-17F and TNFSF13β ($p<0.05$). However, both genotypes show different expression patterns where IBS002 caused a more rapid up-regulation of CXCLi2, IFN-γ, IL12α, IL-18, IL-1β, iNOS and IL-10 at 3 days post-infection (DPI), meanwhile the expression of CCL4, CXCLi1, IFN-γ, IL-12α, IL-1β and iNOS genes were significantly higher in AF2240 compared to IBS002 at 4 DPI. In addition, the expression of IL-10 was significantly higher in IBS002 infected chickens at 3 and 4 DPI compared to AF2240 infected chickens. Hence, infection with velogenic genotype VII and VIII NDV induce cytokines and chemokines associated with inflammatory reactions. Both the expressions of IFN-γ and CXCLi2 transcripts were up-regulated in $CD4^+$ T cells of AF2240 and IBS002 infected chickens. However, IBS002 showed significantly higher up-regulation of CXCLi2 at day 1 and 3 post-infection compared to AF2240. Furthermore, the up-regulation of IL-18 was readily detectable in IBS002 infected $CD4^+$ T cells at day 1 post-infection. In conclusion, the current study demonstrated the differences in the immunophenotyping of B, T and macrophage populations as well as the expressions of cytokines, chemokines and immune-related genes expression in chickens infected with different genotypes of velogenic NDV strains and vvIBDV. The findings from this study are of valuable information for future study in understanding the immunopathology of the respective virus infection and vaccine –induced immunity.

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

TINDAK BALAS DAN REGULASI SISTEM IMUN SEMASA JANGKITAN VIRUS PENYAKIT BURSA BERJANGKIT DAN VIRUS PENYAKIT SAMPAR PADA AYAM

Oleh

MEHDI RASOLI PIROZYAN

Jun 2013

Pengerusi : Profesor Abdul Rahman Omar, PhD

Fakulti: Institut Biosains

Penyakit bursa berjangkit (IBD) adalah disebabkan oleh virus IBD (IBDV), virus limfotropik yang sangat berjangkit yang mengaruh kesan sitosidal pada limfosit B dalam bursa Fabricius. Oleh sebab itu, penyakit ini telah dianggap sebagai penyakit yang mempunyai impak yang besar ke atas ayam muda akibat daripada kesan imunotindasan. Walau bagaimanapun, kesan virus ke atas fungsi limfosit bukan B dan rembesan sitokin dan kemokin tidak dikaji dengan teliti. Manakala, virus penyakit sampar (NDV) adalah virus sangat berjangkit yang telah menyebabkan kerugian ekonomik yang besar kepada industri poultri. Virus ini boleh diklasifikasikan kepada beberapa genotip, namun, NDV velogenik adalah tergolong dalam genotip V, VI, VII, VIII dan X. Oleh sebab itu, pemahaman berkenaan imunoregulasi IBDV dan NDV terhadap sistem imun perumah dapat memberikan maklumat penting dalam menentukan imunopatologi virus berkenaan.

Dalam kajian ini, imunofenotip limfosit dan penghasilan ekspresi sitokin dan kemokin telah dinilai menggunakan kaedah aliran sitometri dan GeXP/PCR masa-

nyata, masing-masing, untuk memahami peranan sel B, T dan makrofaj semasa jangkitan IBDV and NDV. Berdasarkan kajian *in vitro,* IBDV sangat virulen (vvIBDV) strain UPM0081 dapat dikesan dalam sel titisan monosit-makrofaj, sel HD11 seawal 6 jam selepas jangkitan. Manakala, sel titisan T $CD4^+$ dan $CD8^+$ ayam, ConA-C1-Vick tidak bertindak balas terhadap jangkitan *in vitro* IBDV. Kesan sitosidal *in vitro* IBDV terhadap sel titisan HD11 menunjukkan berlakunya apoptosis di mana 6% daripada sel mengalami apoptosis awal pada 24 jam diikuti dengan 11% daripada sel yang mengalami apoptosis lewat. Peningkatan kawal-atur sitokin dan kemokin berkaitan pro-keradangan dan gen lain yang berkaitan seperti CXCLi1, CXCLi2, CCl4, IL12α, IL-18, IL-1β, iNOS, TLR-3 dan MHCI dikesan dalam sel titisan HD11 yang dijangkiti IBDV. Dalam kajian *in vivo*, IBDV dapat dikesan dalam limpa dan bursa seawal 2 hari selepas jangkitan vvIBDV (UPM0081) pada ayam bebas patogen khusus (SPF). Namun, penyusupan populasi makrofaj $Kul1^+$ dalam limpa dan bursa adalah berbeza dalam kajian ini. Ekspresi sitokin, kemokin dan gen berkaitan imun yang lain dalam limpa dan bursa dibandingkan dalam kajian ini untuk memahami patogenesis jangkitan vvIBDV. Walau bagaimanapun, hanya ekspresi TLR-3 dan TLR-7 menurun pada hari ke-3 selepas jangkitan pada limpa dan bursa. Dalam kajian ini, IL-10 sitokin anti-inflamasi yang biasanya dirembeskan oleh makrofaj, menurun ekspresinya dalam sel HD11 tetapi tiada perubahan ketara pada bursa dan limpa ayam selepas jangkitan. Kesan ini boleh menjejaskan fungsi sel B dalam limpa dan juga bursa yang dijangkiti IBDV. Penyusupan makrofaj ke dalam bursa diperhatikan pada hari ke-4 selepas jangkitan. Berbeza dengan peningkatan yang berterusan IL-6, IL-12α, dan iNOS dalam bursa, tahap tertinggi IL-6 dan IL-12α ditemui dalam limpa pada hari ke-2 selepas jangkitan manakala iNOS dicatatkan dengan rembesan tertinggi dalam limpa pada hari ke-3 selepas jangkitan. Tambahan

pula, IL-8 (CXCLi2) dan IL-18 masing-masing mencatatkan tahap rembesan tertinggi pada hari ke-2 dan ke-3 selepas jangkitan, masing-masing dalam limfa dan bursa.

Dalam kes NDV, imunregulasi NDV velogenik genotip VII (IBS002) dan VIII (AF2240) pada makrofaj, limfosit B dan T ayam semasa jangkitan akut virus tersebut dalam ayam SPF telah dicirikan. Kedua-dua genotip NDV mengaruh penurunan drastik limfosit $CD4^+$ dan $CD8^+$ dan berkaitan dengan penyusupan makrofaj dalam limfa pada hari ke-3 selepas jangkitan. Punca utama susutan limfosit T mungkin disebabkan oleh proses apoptosis kerana NDV menyebabkan 37.1% dan 39% sel ConA-C1-Vick mengalami apoptosis pada 24 dan 48 jam selepas jangkitan. Tambahan lagi, profil ekspresi gen menunjukkan peningkatan kawal-atur CCL4, CXCLi1, CXCLi2, IFN-γ, IL12α, IL-18, IL-1β, IL-6, iNOS, TLR-7, MHCI, IL-17F dan TNFSF13β ($p < 0.05$). Walau bagaimanapun, kedua-dua genotip menunjukkan corak ekspresi yang berbeza di mana IBS002 menyebabkan peningkatan kawal-atur CXCLi2, IFN-γ, IL12α, IL-18, IL-1β, iNOS dan IL-10 menjadi lebih tinggi pada 3 hari selepas jangkitan (DPI), manakala, ekspresi daripada CCL4, CXCLi1 , IFN-γ, IL-12α, IL-1β dan iNOS adalah lebih tinggi pada AF2240 jika dibandingkan dengan IBS002 pada 4 DPI. Di samping itu, ekspresi IL-10 adalah jauh lebih tinggi dalam ayam yang dijangkiti IBS002 pada hari ke-3 dan ke-4 selepas jangkitan. Oleh itu, jangkitan dengan genotip velogenik VII dan VIII NDV mengaruh sitokin dan kemokin yang berkait rapat dengan tindak balas keradangan. Ekspresi kedua-dua transkrip IFN-γ dan CXCLi2 pada sel subset T $CD4^+$ meningkat kawal-aturnya dalam ayam yang dijangkiti AF2240 dan IBS002. Walau bagaimanapun, IBS002 menunjukkan kawal-atur CXCLi1 tinggi yang ketara pada hari pertama dan ketiga

selepas jangkitan jika dibandingkan dengan AF2240. Tambahan pula, kawal-atur IL-18 mudah dikesan dalam sel T $CD4^+$ yang dijangkiti dengan IBS002 pada hari pertama selepas jangkitan. Secara kesimpulan, kajian ini menunjukkan perbezaan pada imunofenotip populasi makrofaj, B dan T serta ekspresi sitokin, kimokin dan gen lain berkaitan imun dalam ayam yang dijangkiti genotip velogenik berlainan NDV dan vvIBDV. Penemuan kajian ini membawa kepada maklumat berguna untuk kajian masa hadapan dalam kefahaman imunopatologi jangkitan virus tersebut dan keimunan aruhan vaksin.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisor Prof. Dr. Abdul Rahman Omar and co-supervisors Prof. Datin Paduka Dr. Aini Ideris, Prof. Dr. Mohd Hair Bejo, Prof. Dr. Pete Kaiser and Dr. Sheau Wei Tan for their unwavering support and advice. I am immensely grateful for the opportunities you have given me while I am studying under your kind supervision over the last few years, including your encouragement to communicate my research with others world-wide and broaden my research network. Many thanks for your supervision and input.

I wish to acknowledge the guidance and support from Dr. Yeap Swee Keong, Dr. Hassan Moeini, Dr. Delphine Beeckman, Dr. Lisa Rothwell and Dana Chow. My gratitude also goes out to my colleagues and lab mates; Siamak Ebrahimi, Kiarash Roohani, Mozhgan Mollayee, Dr. Davoud Jazayeri, Farhana Zaini, Kristeen Wen, Aimi, Amanda Teoh, Mostafa and Farah who have always supported me emotionally and technically. Moreover, I would like to show my appreciation to all the staff of Institute of Bioscience, especially staff at Laboratory of Vaccines and Immunotherapeutics for their support and kindness. I wish to extend my appreciation to everyone, although not individually named here, who had contributed directly or indirectly to my project and thesis. I would like to thank the Ministry of Higher Education (MOHE), Government of Malaysia for providing Institute of Bioscience, Higher Institution Centre of Excellence (IBS HICoE) grant and Universiti Putra Malaysia for providing Graduate Research Fellowship for supporting my study. Finally, I am indebted to my parents, my sisters and brother in laws for their love, moral support and understanding and without their emotional support, this work would not have been possible.

I certify that an Examination Committee met on 17th June 2013 to conduct the final examination of Mehdi Rasoli Pirozyan on his Doctor of Philosophy thesis entitled "Immune system regulation and response during infectious bursal disease virus and Newcastle disease virus infections in chickens" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the Degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

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

MEHDI RASOLI PIROZYAN

Date: 17th June 2013

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LIST OF ABBREVIATIONS

Ab	Antibody
bp	Base pair
BSA	Bovine serum albumin
CaCl	Calcium chloride
CD	Cluster of differentiation
cDNA	Complementary DNA
Con-A	Concanavalin A
CSF	Colony-stimulating factor
DMEM	Dulbecco's modified eagle media
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
ddH_2O	Double Distilled Water
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EID_{50}	50% Egg Infective Dose
FBS	Fetal bovine serum
FITC	Fluorescein
g	Gram
g	Gravity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBSS	Hanks balance salt solution

IC_{50}	Inhibition concentration that reduces 50% of cell viability compared to control
i.e.	In example
IFN	Interferon
IgM	Immunoglobulin M
IL	Interleukin
iNOS	Inducible nitric oxide synthases
kDa	Kilo Dalton
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mL	Milliliter
MOI	Multiplicity of infection
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium Iodide
PGE2	Prostaglandin E2
qPCR	Quantitative polymerase chain reaction
RPM	Round per minute
SD	Standard deviation

SDS	Sodium lauryl sulfate
ssRNA	Single stranded RNA
TAE	Tris-Acetate-EDTA
Taq	Thermus aquaticus
Tm	Melting Temperature
Th	T helper
TLR	Toll-like receptor
TMB	3,3',5,5' tetramethylbenzidine
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
UPM	Universiti Putra Malaysia
USA	United State of America
UV	Ultraviolet
w/v	Weight/Volume
v/v	Volume/Volume
α	Alpha
β	Beta
γ	Gamma
µ	Micro
µl	Micro liter
µ	Micro Molar
°C	Celsius

CHAPTER 1

INTRODUCTION

Infectious bursal disease virus (IBDV) and Newcastle disease virus (NDV) are among the highly infectious virus of chickens, which contribute to the major causes of economic losses in poultry industry. IBDV is a bisegmented, double stranded RNA virus that belongs to the family *Birnaviridae*. IBDV infection associated with acute infection in susceptible birds that often associated with severe immunosuppression. Both classical and virulent strains of IBDV are able to infect IgM^+ B lymphocytes. However, a study conducted by Rauf et al. (2011b) showed that classical IBDV (cIBDV) produced more prominent bursal damage and caused infiltration of T cells and inflammatory response compared to variant (vIBDV). The same study illustrated that expression of IFN-α, IFN-β, IL-6 and iNOS differ in cIBDV and vIBDV infected bursas. Moreover, expression of chemokines such as CXCLi2 and MIP-α was higher in cIBDV, indicating that bursal lesions, infiltration of T cells and expression of different cytokines varies in different strains of IBDV infected chickens. Ruby et al. (2006) studied the changes in gene expression in bursa of genetically resistant and sensitive inbred lines of SPF chickens by means of microarray, where the gene expressions for inflammatory, proinflammatory and chemokines genes were different in both lines following infection with classical strain of IBDV.

During the acute phase, replicating of virus in different tissues especially in the bursa of Fabricius causes extensive destruction of bursal follicles (Tanimura and Sharma, 1997) resulting in diminution of circulating IgM^+ B cells (Hirai et al., 1981; Rodenberg et al., 1994). Influx of T cells is another phenomenon in infected bursa (Kim et al., 1999; Kim et al., 2000; Sharma et al., 2000; Tanimura and Sharma, 1997). Although IBDV causes activation and proliferation of bursal T cells, there are evidence indicating that T cells are not target of infection and replication by IBDV (Kim et al., 2000). However, numerous studies have indicated that macrophages and monocytes are susceptible to infection with IBDV (Burkhardt and Müller, 1987; Inoue et al., 1992; Käufer and Weiss, 1976; Käufer and Weiss, 1980; Komine et al., 1989; Müller, 1986).

Previous studies have indicated that IBDV up-regulates expression of Th1-like and proinflammatory cytokines such as IFN-γ, IL-18, IL-1β and IL-6 in bursa (Eldaghayes et al., 2006). Additionally, IBDV increase the expression level of IFN-γ in infected spleens (Khatri and Sharma, 2008). Regardless of previous studies that have been conducted to understand the functions of macrophages in pathogenesis of IBD, there is still lack of information on the effects of vvIBDV interaction with macrophages and T cells. Moreover, this study intended to examine various cytokines, chemokines and other immune-related genes during *in vitro* and *in vivo* IBDV infections in transformed chicken macrophage-like cell line (HD11), transformed chicken T cell line (ConA-C1-Vick) and specific-pathogenic-free (SPF) chickens.

Newcastle disease (ND) is an infectious, highly contagious and pathogenic avian viral disease caused by a *paramyxovirus*. Since 1960 the majority of virulent NDV circulating throughout the world are of genotypes V, VI, VII, VIII and X (Miller et al., 2010). Since 2000 until present, genotype VII is the predominant NDV strains circulating in various parts of the world (Aldous et al., 2003; Wang et al., 2006). In addition, the majority of the outbreaks are reported in NDV vaccinated flocks. Studies have been carried out to determine the possible factors contributed to the ability of the virus to break vaccine-induced immunity.

Previous studies have illustrated that NDV induces up-regulation of various cytokines such as IFN- γ, IL-6 and iNOS in spleen (Ecco et al., 2011; Rue et al., 2011). Moreover, *in vitro* studies have illustrated that NDV can cause an up-regulation of IFN-β which is an antiviral interferon (Krishnamurthy et al., 2006). It has been also stated that NDV can increase the expression level of IL-16, IL-18, IFN-γ and IFN-α in peripheral blood of chickens (Liu et al., 2012). However, the interaction of virulent NDV with different lymphocytes and cytokine responses are still unclear. Understanding the interaction of virulent IBDV and NDV with different immune cells and the ability of the respective viruses to regulate the chicken immune system will provide valuable information to define the molecular immunopathology of NDV and IBDV associated diseases.

The hypotheses of this study are: Very virulent IBDV able to activate and differentially regulate the lymphocyte-monocyte populations in bursa and spleen of infected specific-pathogen-free (SPF) chickens that associated with up-regulation of proinflammatory related cytokines and chemokines. Second hypothesis is different

genotypes of velogenic NDV differentially modulated lymphocyte-monocyte responses and proinflammatory cytokine expressions in spleen of infected SPF chickens.

Hence, the objectives of this study were:

a) to develop a quantitative multiplex GeXP assay that is able to detect chicken cytokines, chemokines and other immune-related genes.
b) to assess the ability of a very virulent strain of IBDV to infect non- B cell chicken cells (chicken monocyte/macrophage HDII cell line and ConA-C1-Vick T cell line).
c) to analyse the *in vitro* modulation effect of very virulent IBDV strain on cytokines, chemokines and other immune-related gene expressions of HD11 cells.
d) to evaluate the *in vivo* immunoregulatory effect of very virulent IBDV strain on different populations of lymphocytes and expression patterns of cytokines, chemokines and immune-related genes in the spleen and bursa of Fabricus of infected SPF chickens.
e) to compare the *in vivo* immunoregulatory effect of two different genotypes of velogenic NDV, genotype VIII strain AF2240 and genotype VII strain IBS002 on different populations of lymphocytes and expression patterns of cytokines, chemokines and immune-related genes in the spleen of infected SPF chickens.
f) to determine the expression of selected cytokines and chemokines from purified $CD4^+$ splenic T cells obtained from SPF chickens infected with NDV strains AF2240 and IBS002.

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LIST OF PUBLICATIONS

1- M. Rasoli, S.K. Yeap, S.W. Tan, P. Kaiser, A. Ideris, M.H. Bejo, N.B.M. Alitheen, S. Ebrahimi, Y.W.T. Kristeen, H. Moeini, A. Zulfadli Jamil and A.R. Omar. Immune regulation effects of genotype VII and VIII virulent Newcastle disease viruses. Submitted to journal of comparative immunology, microbiology and infectious disease.

2- S. Ebrahimi Nigjeh, F. Md Yusoff, N.B.M. Alitheen, M. Rasoli, S. K. Yeap and A. R. Omar. Cytotoxic effect of ethanol extract of microalga, Chaetoceros calcitrans and its mechanisms in inducing apoptosis in human breast cancer cell line. Journal of Biomedicine and Biotechnology. 2013. doi.org/10.1155/2013/783690.

3- H.Y. Lam, S.K. Yeap, M. Rasoli, A.R. Omar, K. Yusoff, S. Abd-Aziz, and N. Alitheen. Safety and Clinical Usage of Newcastle Disease Virus in Cancer Therapy. Journal of Biomedicine and Biotechnology 2011. doi:10.1155/2011/718710.

4- B. Jalilian, A.R. Omar, M.H. Bejo, N.B. Alitheen, M.Rasoli, S. Matsumoto. Development of avian influenza virus H5 DNA vaccine and MDP-1 gene of Mycobacterium bovis as genetic adjuvant. Genetic Vaccine and Therapy 2010, 8:4 doi:10.1186/1479-0556-8-4.

5- M. Rasoli, A.R. Omar, I. Aini, B. Jalilian, Sh. Syed Hassan, M. Mohamed. Fusion of HSP70 gene of Mycobacterium tuberculosis to hemagglutinin (H5) gene of avian influenza virus in DNA vaccine enhances its potency. Acta virologica 54: 33 – 39, 2010. doi:10:4149/av_2010_01_33.

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7- M. Rasoli, S.W. Tan, S.K. Yeap, A. Ideris, M.H. Bejo, P. Kaiser, A.R. Omar. Virulent Newcastle disease virus differentially modulates chicken splenic lymphocytes/macrophage population and cytokines expression. (Poster presentation). In; proceeding of XII Avian immunology Research Group Meeting, The Roslin institute, The University of Edinburgh. (2012) pp 83.

8- A.R. Omar, M. Rasoli, H. Moeini, S.K. Yeap, P. Kaiser, M.H. Bejo, A. Ideris, S.W. Tan. Characterization on the role of non B lymphocytes during infectious bursal disease virus infection. In; proceeding of XII Avian immunology Research Group Meeting, The Roslin institute, The University of Edinburgh. (2012) pp 28. (Oral Presentation).

9- M. Rasoli, A.R. Omar, I.Aini,., B. Jalilian, Sh.Syed Hassan, M.Mohamed, Enhancement of DNA vaccine potency through linkage of M. tuberculosis HSP70 gene to Avian influenza virus H5 gene. In; Proceedings of the 21st Veterinary Association Malaysia Scientific Congress,The Legends Port Dickson, Negri Sembilan. (2009) pp126. (Oral Presentation).

10- B. Jalilian, A.R. Omar, M.H. Bejo, N. Alitheen, M. Rasoli, M. Abdul-Razak. Enhancement of antibody responses in chickens vaccinated with DNA plasmid constructs of H5 gene of avian influenza virus and MDP-1 gene of Mycobacterium bovis. In; Proceedings of the 21st Veterinary Association Malaysia Scientific Congress, the Legends Port Dickson, Negri Sembilan. (2009) pp121. (Oral Presentation).