UNIVERSITI PUTRA MALAYSIA

CHICKEN TRANSCRIPTOME ANALYSIS AND DISCOVERY OF NOVEL GENES INVOLVED IN IBDV INFECTIOUS BURSAL DISEASE VIRUS INFECTION

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By

SHARANYA RAVI

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

January 2018

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DEDICATION

I would like to dedicate this entire work to my husband for being the pillar of my strength and has given his complete support throughout this journey. I would also like to dedicate this to my son for being patient with me and supporting me in his own way.

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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January 2018

Chairman : Nurulfiza Mat Isa, PhD
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Infectious bursal disease (IBD) is a major concern to food security because pathogenic strain of IBD virus (vvIBDV) can cause high mortality and immunosuppression in chickens that has not been controlled till date. This study aims to study the differential expression and discover novel genes in IBDV infected chickens. 18 transcriptomes generated from *de novo* sequencing of samples from six different lines of IBDV infected and control chicken were analysed. Sequences that did not map to the reference genome of *Gallus gallus* were analysed for differentially expressed genes (DEG) in IBDV infected chickens. About 600 unigenes out of 10,828 were selected for VENNTURE (Venn diagram) analysis that resulted in 12 upregulated and 18 downregulated DEGs that were common to all the six lines of chicken. Annotation predicted the functions to be involved in the transcription factor activities and extra cellular binding activities which aids in the immune response. Three upregulated and four downregulated unigenes did not have any significant BLAST hits. Gene networks generated using the weighted gene correlation network analysis (WGCNA) predicted the functions of the unknown sequences to be related to interleukin 18 binding protein, mucin13 isoform XI and extracellular matrix protein, cerebellar degenerative protein, cell surface protein, ubiquitin conjugating protein. Quantitative PCR was performed on 10 genes including the unknown genes (7 genes), FoxP3 gene and two known genes that were homologues with species other than *Gallus gallus* to validate the differential expression. Presence of FOX P3 gene was validated by visualizing the amplification. Based on the results, four out of seven unknown were found to be in chicken and their functional prediction contributes to the resistance against the disease.

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

ANALISIS TRANSKRIPTOM AYAM DAN PENEMUAN GEN NOVEL TERLIBAT DALAM JANGKITAN IBDV

Oleh

SHARANYA RAVI

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Penyakit bursal berjangkit (IBD) adalah sangat penting dari sudut ekonomi bagi industri makanan dan menjadi kebimbangan utama dalam isu keselamatan makanan. Strain sangat patogenik virus IBD (vvIBDV) pertama kali dilaporkan di Eropah pada tahun 1980-an dan ia menyebabkan kematian yang tinggi serta kemerosotan sistem imun pada ayam. Penyebaran penyakit tersebut masih belum dikawal sepenuhnya walaupun kajian penyelidikan telah dijalankan bertahun lamanya. Oleh itu, kajian ini dijalankan bertujuan untuk melihat gen novel yang boleh membantu penentangan penyakit atau dapat memberikan lebih banyak maklumat tentang kerintangan ayam. Sebanyak 18 transkriptom dari enam titisan sel ayam yang berbeza bagi ayam kontrol dan dijangkiti IBDV telah digunakan untuk analisis. Data mentah yang diperolehi melalui penjujukan Illumina tertakluk kepada pemprosesan hiliran khas kawalan mutu, pemetaan rujukan dan perhimpunan *de novo* bagi jujukan yang tidak dapat dipetakan. Set jujukan yang tidak dipetakan kepada genom rujukan *Gallus gallus* dianalisis untuk pengekspresan gen berbeza akibat jangkitan IBDV. Daripada 10,828 unigenes yang tidak dipetakan dengan genom rujukan, jujukan yang paling signifikan (log 2 perubahan kali ganda <-2 atau> 2) telah ditapis (~ 600 jujukan nukleotida). Untuk mengelakkan jujukan yang bertindih bagi enam titisan sel tersebut, analisis VENNTURE (Venn Diagram) digunakan. Ini menghasilkan 12 jujukan nukleotida mengalami ekspresi meningkat dan 18 jujukan nukleotida mengalami ekspresi menurun yang hadir bagi kesemua enam titisan sel ayam. Setelah anotasi dilakukan, terdapat tiga jujukan unigenes mengalami ekspresi meningkat dan empat jujukan unigenes mengalami ekspresi menurun yang tidak mempunyai hits BLAST yang signifikan. Analisis istilah ontologi gen (GO) mendedahkan bahawa jujukan ini kebanyakannya terdapat di rantau nukleus. Fungsi utama mereka adalah dalam aktiviti faktor transkripsi khususnya membantu dalam tindak balas imun dan aktiviti pengikatan sel luaran.

Untuk memahami fungsi jujukan yang tidak diketahui, analisis rangkaian gen dilakukan menggunakan perisian R rangkaian korelasi gen yang tertimbang (WGCNA), di mana ia melihat gen yang paling berkorelasi dan mengelompokkannya ke dalam modul. Ini menghasilkan ramalan fungsian bagi tiga jujukan yang mengalami ekspresi meningkat dikaitkan dengan protein pengikatan interleukin 18, mucin13 isoform XI dan protein matriks ekstraselular; manakala empat gen yang mengalami ekspresi menurun adalah berkaitan dengan protein degeneratif cerebellar, protein sel permukaan dan protein ubiquitin berkonjugat. PCR kuantitatif telah dilakukan pada 10 gen iaitu tiga gen yang tidak diketahui yang mengalami ekspresi meningkat dan empat gen yang tidak diketahui yang mengalami ekspresi menurun, gen FoxP3 dan dua gen yang diketahui yang mempunyai homolog dengan spesies lain selain daripada *Gallus gallus.* Ujian ini mengesahkan gen pengekspresan berbeza bagi gen yang mengalami ekspresi meningkat dan gen yang mengalami ekspresi menurun pada fasa kontrol dan jangkitan. Keputusan gen yang mengalami ekspresi menurun terbukti konsisten dengan ramalan yang dibuat melalui analisis penjujukan. Walau bagaimanapun, ujian pengesahan bagi gen yang mengalami ekspresi meningkattelah menunjukkan keputusan yang sebaliknya bila dibandingkan dengan sel ayam kontrol. Dua daripada gen yang dipilih untuk untuk ujian pengesahan tidak diamplifikasi sekaligus mencadangkan ralat semasa penjujukan atau primer yang tidak spesifik. Menariknya, FOX P3 gen yang sebelum ini tidak diketahui hadir pada *Gallus gallus* telah didapati hadir dan disahkan melalui amplifikasi PCR. Kesimpulannya, empat daripada tujuh jujukan yang tidak diketahui telah terbukti hadir dan ditemui dalam ayam bersama dengan ramalan fungsian mereka yang menyumbang kepada ketahanan terhadap penyakit.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

AC-ELISA	Antigens Capture ELISA
AGID	Agar Gel Immunodiffusion
APS	Adenosine 5 phosphosulfate
B2G	Blast2GO
BALT	Bronchial lymphoid tissue
BLAST	Basic Local Alignment Search Tool
BP	Biological process
BT	Broiler Type
CALT	Conjunctiva Lymphoid Tissue
CSH	Cross Species Hybridization
Ct	Cycle Threshold
DNA	Deoxyribo Nucleic Acid
dpi	Day Post Infection
dsRNA	Double Stranded RNA
ELISA	Enzyme Linked Immunosorbent Assay
FKPM	Fragments per Kilobase of Transcript per Million Mapped Fragments
FVM	Faculty of Veterinary Medicine
GALT	Gut-Associated Lymphoid Tissue
GO	Gene Ontology
HCMV	Human Cytomegalovirus
HI	Haemagglutination Inhibition
HIV	Human Immunodeficiency Virus
IBD	Infectious Bursal Disease

IBDV	Infectious Bursal Disease Virus
IFN	Interferon
IHA	Indirect Haemagglutination
IHC	Immunohistochemistry Test
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LT	Layer Type
MDA5	Chicken Melanoma Differentiation-Associated Gene 5
MDV	Marek's Disease Virus
MF	Molecular function
MHC	Major Histocompatibility complex
NCBI	The National Centre for Biotechnology Information
NGS	Next Generation Sequencing
NO	Nitric Oxide
nr	non-redundant
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PPi	Pyrophosphate
RBC	Red Blood Corpuscles
REF	Reference
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism

SOLiD	Sequencing by Oligo Ligation Detection
SPF	Specific-Pathogen-Free
TAR	Target
TGICL	TIGR gene indices clustering tools
UK	United Kingdom
UPM	Universiti Putra Malaysia
VN	Virus Neutralization
vvIBDV	Very Virulent IBDV
WGCNA	Weighted Gene Correlation Network Analysis

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The world has over 23 billion poultry birds—about three per person on the planet (FAOSTAT, 2016) and about five times more than 50 years ago. They are kept and raised in a wide range of production systems, and provide mainly meat, eggs and manure for crop fertilization. Poultry meat and eggs are among the most common animal source of food consumed at global level, through a wide diversity of cultures, traditions and religions, making them the key to food security and nutrition. Within the livestock sector, poultry emerges as the most efficient subsector in its use of natural resources and in providing protein to supply a growing global demand.

Global production of eggs is about 73 million tons and global production of poultry meat is close to 100 million tons (GLEAM 2, 2016). Demand for animal food source is increasing because of population growth; rising income and urbanization, and poultry meat has shown the fastest growth in the last decades. The average annual growth rate over the last five decades was 5%, which was the highest among other sources of animal protein (Alexandratos & Bruisma, 2012).

The growth of the global livestock sector is expected to continue. Global human population is estimated to reach 9.6 billion in 2050, in this context, Alexandratos and Bruisma (2012) projected that the demand for animal source food could grow by 70% between the year 2005 and 2050. Demand for eggs will increase by 65%. Thus, it becomes vital to protect the poultry industry from economic losses due to epidemics (Mottet & Tempio, 2017).

Infectious bursal disease virus (IBDV) that causes the Infectious bursal disease (IBD), otherwise known as the Gumboro disease, belongs to the *Avibirna*virus genus of the *Birnaviridae* family. IBDV typically replicates in the bursa of Fabricius; thus, leading to suppression in both humoral and cellular immunity in chicken that are infected with them. IBD is one of the primary reasons for the reduced productivity and this incurs loss to the poultry industry all over the world, irrespective of the country's development (Shane, 1997). IBD is spread worldwide with two serotypes (Sharma, Kim, Rautenschlein, & Yeh, 2000) (OIE, Terrestrial Manual 2008). Serotype 1 is the one responsible for clinical cases of the disease and many commercial vaccines have been produced for Serotype 1 (OIE, Terrestrial Manual 2008).

The very virulent IBD virus is capable of infecting chickens in the presence of maternally derived or higher levels of vaccinal antibodies causing very high mortalities and bursal damage with severe economic losses (Shane, 1997; Lukert, 1997; Sainsbury, 2000; Islam & Samad , 2004; Mbuko et al. 2010). The chickens are

most susceptible to IBD between the age of 3 and 6 weeks, when the bursa of fabricius is at its maximum rate of development and the bursa follicles are filled up with immature lymphocytes. The IBD virus replicates and cytolytically affects the actively dividing B lymphocytes in the bursa of Fabricius (Baxendale, 1981; Lukert, 1997). Thus, it is very important to look for methods to control the disease.

IBDV enters by the oral route of the host organism (chicken) and is transported to other tissues by the phagocytic cells most probably by the resident macrophages. The virus attacks the actively dividing B cells in the bursa of fabricius that bear the IgM (Rodenberg et al., 1994). The virus destroys the lymphoid follicles in the bursa of fabricius as well as the circulating B cells in the secondary lymphoid tissues such as gut-associated lymphoid tissue (GALT); conjunctiva associated lymphoid tissue (CALT), bronchial associated lymphoid tissue (BALT), caecal tonsils, Harderian gland, etc. Apart from B cells, they infect and replicate in macrophages as well, where they produce pro-inflammatory mediators and cytokines, whose levels rises to the maximum during the early phase of active virus replication (Palmquist et al., 2006).

The draft chicken genome used as a reference sequence was assembled using whole genome shotgun sequencing of DNA from a single inbred female jungle fowl (*Gallus gallus*, the ancestor of domesticated chickens; Fumihito et al., 1994) using fosmid, bacterial artificial chromosome (BAC) and plasmid (Hillier et al., 2004). In birds, female is the heterogametic sex, with single copies of the Z and W chromosomes. Thus, the final assembly had very poor representation of these chromosomes. Unlike the rest of the genome, the W chromosome has a high repeat content thus; only minimal sequence assembly was possible. About 5%–10% of genes were missing from the final assembly. This could have been due to the possibility of gene duplications and GC-rich sequences. Consequently, it becomes important to complete the chicken genome sequence to a high quality for comparative genomics and gene discovery. Moving forward, there is a high demand for food that is safe to consume, Thus, raising the demand for livestock that has been treated with lesser chemicals and antibiotics. Discovery of disease resistant traits using conventional genetic selection is both time consuming and costly; hence, studying the chicken genomics aids in finding a solution to this problem (Burt, 2005).

1.2 Statement of problem

Vaccination is the main strategy used to contain IBD. However, IBD outbreaks still occur in chicken farms worldwide due to the emergence of variant strains of IBDV and very virulent IBDV (Lee, Kim, Wu, & Lin, 2015). As a result, it would be a vital analyse the transcriptomes of the infected and the uninfected chickens to compare and understand the different RNA contents in both states and use the differences to improve resistance.

1.3 Justification of research

Understanding the expression of the genes in the system, especially those that are affected during a disease condition and are involved in the disease could pave path to develop therapeutic strategies to combat the disease. High-throughput RNA sequencing (RNA-seq) technology, a powerful way to profile the transcriptome with great efficiency and higher accuracy, has been employed in various viral infections and disease. RNA-seq technology has the potential to reveal the alterations of the dynamics of the genome of the pathogen itself and the systemic change in host gene expression in the process of infection by pathogens, which could help uncover the pathogenesis of the infection. Previous study comparing the gene expression in chicken under the influence of two different viral infection caused by influenza viruses H5N8 and H1N1 showed that the transcriptomic data of the host could reveal the underlying genes involved by analysing their expression levels at two different conditions (Park et al., 2015).

1.4 Research hypotheses

Transcriptomic data obtained from RNA seq analysis of infected and uninfected states of six different lines of chicken will help complete the missing parts of the *Gallus gallus* genome and differential expression profiles would reveal genes that play a vital role in the IBDV infection. Thus, they can give better understanding of the pathogenesis leading to better treatment against the disease.

1.5 Research objectives

1.5.1 General objective

To analyse the differential expression of *de novo* assembled transcriptome and discover novel genes present in control and IBDV infected chickens.

1.5.2 Specific objectives

1. To annotate the transcribed genes of *de novo* assembled transcriptome dataset from six different lines of control and IBDV infected chickens.
2. To investigate the differential gene expressions of the annotated genes;
3. To perform gene network analysis for function prediction of novel differentially expressed genes;
4. To validate the genes using quantitative PCR.

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