Investigation of the molecular pathogenesis of newly emerged Newcastle disease virus in Indonesia

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Acronyms

ACIAR	Australian Centre For International Agricultural Research
AGT	Angiotensinogen
ANOVA	One-Way Analysis Of Variance
ARRIVE	Animal Research Reporting Of In Vivo Experiments
BHI	Brain–Heart Infusion
BLAST	Basic Local Alignment Search Tool
BLS3	Biosafety Level 3
CAT	Catalases
cDNA	Complementary DNA
CEFs	Chick Embryo Fibroblasts
CPM	Count Per Million
CT	Cycle Threshold
DDR	DNA Damage Response
DEG	Differentially Expressed Genes
DNA	Deoxyribonucleic Acid
DPI	Day Post Infection
F	Fusion
FAO	Food And Agriculture Organization
FDR	False Discovery Rate
FFPE	Formalin-Fixed Paraffin-Embedded
GPx	Glutathione Peroxidase
GPx	Glutathione Peroxidase
H&E	Hematoxylin & Eosin
HA	Haemagglutination

HN	Hemagglutinin-Neuraminidase
IBDV	Infectious Bursal Disease Virus
IFIT5	Tetratricopeptide Repeats 5
IHC	Immunohistochemistry
IPA	Ingenuity Pathway Analysis
LFC	Lof Fold Change
lncRNA	Long Non-Coding RNA
M	Matrix
MDT	Mean Death Time
mRNA	Messenger Ribonucleic Acid
NCBI	National Center For Biotechnology Information
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NDV-GVII	Genotype VII Of NDV
NHMRC	National Health And Medical Research Council
NO	Nitric Oxide
NP	Nucleocapsid Protein
O2−	Superoxide Anion
OIE	The World Organization For Animal Health
OxRADD	Oxidative Repair Assisted Damage Detection
P	Phosphoprotein
PC3	Physical Containment Level
PCR	Polymerase Chain Reaction
PKR	Protein Kinase R
qPCR	Quantitative Polymerase Chain Reaction

RADD	Repair Assisted Damage Detection
RIN	Rna Integrity Number
RIPK1	Receptor Interacting Protein Kinase 1
RIPK3	Receptor Interacting Protein Kinase 3
RNA	Ribonucleic Acid
RNA-Seq	RNA-Sequencing
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SOD	Superoxide Dismutase
SPF	Specific Pathogen Free
TBP	Tata-Box Binding Protein
TMM	Trimmed Mean Of M Values
TNF	Tumor Necrosis Factor
USA	United States Of America
YWHAZ	Tryptophan 5-Monooxygenase Activation Protein Zeta

Abstract

Newcastle Disease Virus (NDV) has caused significant outbreaks in South-East Asia, particularly in Indonesia. Australia is currently free from virulent strains of NDV. However, the introduction of virulent NDV strains is a severe risk for the Australian poultry industry. Indonesia is the closest country to Australia and spillover might occur through migratory birds. Two viral strains from recent outbreaks have been isolated and analyses by full genome sequencing and phylogenetic assays.

This study was conducted to determine the pathogenesis, candidate genes, biological pathways and tissue tropism of recently emerged genotype VII of NDV (NDV-GVII) viral strains. Chickens were experimentally infected with live NDV-GVII virus. Tissue samples were collected after euthanasia of birds. A transcriptomic analysis based on RNA sequencing (RNA-Seq) of spleen was performed in chickens challenged with NDV-GVII and a control group. Repair Assisted Damage Detection (RADD), and immunohistochemistry staining of Viral HN, caspase-3 and MLKL antigens were employed to analyse DNA damage levels, viral load, apoptosis and necroptosis markers, respectively.

Phylogenetic study results revealed that these two strains were identical and belong to genotype VII.1, class II cluster of avian paramyxoviruses, and have significant differences in amino acid identities with La Sota strain.

In total, 6361 genes were differentially expressed that included 3506 up-regulated genes and 2855 down-regulated genes. Real-time PCR of ten selected differentially expressed genes (DEGs) from the RNA-Seq results showed agreement between the two technologies in detecting DEGs as the correlation between them is 0.98. Functional and network analysis of DEGs showed down regulation of *EIF2* signalling, mTOR signalling, the proliferation of lymphatic system cells, signalling by Rho family GTPases and synaptogenesis signalling in spleen. We have also identified increased expression of *IFIT5, PI3K, AGT* and *PLP1* genes in NDV-GVII infected chickens. Bursal atrophy was associated with profound expression of MLKL and only patchy distribution of viral antigen, providing evidence that the mechanism of lymphoid depletion involved a non-apoptotic pathway of programmed cell death termed necroptosis. RADD and oxididative RADD analysis of bursa showed a DNA damage pattern consistent with the programmed cell death rather than necrosis, consistent with MLKL stain results. MLKL expression in the spleen was less pronounced and largely restricted to the central portion of periarteriolar lymphoid sheaths, while other regions of white pulp expressed neither MLKL nor caspase-3. A shift in tissue tropism from neurologic and gastrointestinal to the immune system compared to previously reported NDV-GVII strains was also observed in this strain.

Significant differences in amino acid identities of circulation viruses and La Sota strain as the most common vaccine strain used in Indonesia shed more light on the probable reason for vaccine failure against these NDV strains and highlights the urgent need for updated vaccine development strategies in South-East Asia. Our findings in activation of autophagy-mediated cell death, lymphotropic and synaptogenesis signalling pathways provide new insights into the molecular pathogenesis of this newly emerged NDV-GVII. This study is the first report of using RADD assay for DNA damage analysis in NDV infection and revealed the persistence of oxidative lesion in the genome after viral challenge. Together with observations of karyorhexis, fibrinous inflammation, and RADD analyses, we conclude that necrosis was responsible for the majority of lymphoid depletion in the spleen. Therefore, we speculate that the progression of NDV infection may deplete various subsets of lymphocytes by different mechanisms.

Declaration

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Mohammad Rabieifaradonbeh

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Thesis structure:

This thesis is presented as a 'Thesis by Publication' and includes a combination of published and submitted manuscripts under review. Each chapter forms a separate scientific manuscript. In consequence, some repetition between the chapters exists in their introduction and or methods sections. For consistency, I have standardised chapter formatting throughout this thesis. The manuscript's current status in the publication process mentioned in the ‘Statement of Authorship' associated with each chapter. All references to manuscripts/chapters of this thesis reflect the current status of that manuscript in the publication process (in press or in review) at the time of thesis submission. Chapter 1 of this thesis (Introduction) reviews the current literature of Newcastle Disease Virus (NDV) and particular genotype VII of NDV in Indonesia, RNA-sequencing, and more, including a summary of research into the determination of molecular pathogenicity of NDV. Chapter 2, entitled “Genome Sequences of Newly emerged Newcastle Disease Virus Strains Isolated from Disease Outbreaks in Indonesia”, has been published in Microbiology Resource Announcement journal. Chapter 3 entitled “Indicators of the molecular pathogenesis of virulent Newcastle disease virus in chickens revealed by transcriptomic profiling of spleen”, is currently under revision in Scientific Reports journal. Chapter 4, entitled “Necroptosis, necrosis, and oxidative DNA damage in lymphoid tissues of chickens infected with genotype VII Newcastle disease virus”, has submitted for publication. Chapter 5 provides the thesis with a final discussion, a summary of all research chapters and recommendation for further research.

1 Introduction

1.1 Newcastle disease

Newcastle Disease (ND) has a worldwide distribution. Because it infects many different avian species, it can be considered a permanent threat to all poultry industries and other aviculture fields (Miller, Patti J et al. 2015). This virus was described for the first time in the island of Java (Indonesia) in 1926, and at the same time in Newcastle (England). Various genotypes have been responsible for different ND panzootics around the world. (Doyle 1927). NDV infects more than 200 bird species (Afonso et al. 2012). Doves, pigeons, and Cormorants are NDV reservoirs. This virus can be transmitted by the feces of infected birds and aerosols via the reservoir birds, movement of equipment and people, contaminated water or feed (Senne 2008). The identification and characterisation of ND are difficult because of a wide range of clinical signs. ND is also known as synonyms like: pseudovogel-pest, pseudo-fowl pest, pseudo-poultry plague, Tetelo disease, avian pest, avian distemper, Korean fowl plague, atypische Geflugelpest, avian pneumoencephalitis, and Ranikhet disease (Senne 2008).

Nearly a century since the discovery of ND, it still has a considerable economic impact on the poultry industry. ND had a worldwide distribution, South America, North America, Asia, Europe, Africa, Oceania and South-East Asia (Palgen et al. 2015), and the high prevalence of ND across the world caused a large economic impact. The impact of ND is very destructive because it causes a very high mortality rate in chickens, and also because of the economic effect from trade restrictions and limitations, The World Organization for Animal Health (OIE) has ranked Newcastle Disease as a list-A disease with other critical diseases such as Avian Influenza (Alexander, D 2000a; Susta et al. 2014). The cost of consistent and effective vaccination is high for the poultry industry. Many smallholder farmers, particularly in developing countries, may not be able to afford the vaccination strategy's operation.

The clinical signs of ND range from asymptomatic to high mortality and are dependent on the strain of NDV (Al-Garib et al. 2003).

1.2 Description of the virus

Newcastle disease virus (NDV) is a member of the genus Avian *orthoavulavirus 1* within the new subfamily *Avulavirinae* of the family *Paramyxoviridae* within the *Mononegavirales* order (Rima et al. 2019b). The family of *Paramyxoviridae* contains other important viruses, including measles, mumps, Sendai virus, Hendra, Nipah, and parainfluenza viruses (Chang & Dutch 2012). NDV has a single-stranded non-segmented negative-sense RNA genome with an envelope. The genome of NDV is approximately 15 kb, and includes six genes 3'-NP-P-M-F-HN-L-5' (Chambers et al. 1986), that encode NDV proteins. The nucleocapsid protein (NP) covers the genomic RNA (Yue et al. 2009). The phosphoprotein (P) and L

protein are essential for the synthesis of NDV (Hamaguchi et al. 1983). The P gene also encodes two other proteins through RNA editing mechanism, the V and W proteins (Steward et al. 1993). The V protein promotes the degradation of STAT1(Parks & Alexander-Miller 2013). The function of W protein has not been completely discovered, but it is probably not involved in the pathogenesis of NDV (Huang, Z et al. 2003). The matrix (M) protein is essential for NDV budding and organises other viral proteins at the cell membrane (Pantua et al. 2007). Hemagglutinin-neuraminidase (HN) protein located on the surface of NDV particles. HN protein has a critical role in attachment and entry of NDV to the host cell and consequently, in the virulence (Huang, Z et al. 2003). The Fusion (F) protein is a glycoprotein found on the surface of NDV. F protein is the major determinant of NDV virulence. The amino acid composition of cleavage site of fusion protein and its susceptibility to host trypsin-like proteases play a significant role in pathogenicity, the spread of infection, and tissue tropism of NDVs (Nagai 1995). The cleavage of F0 protein into F1 and F2 is the initial step of attachment of the virus to the host cell (Senne 2008). Lentogenic NDVs have a monobasic amino acid motif in cleavage site, while the cleavage site of mesogenic and velogenic strains is a multi-basic amino acid motif (Glickman et al. 1988). The furin-like proteases that exist in a wide range of host cells can cleave the F0 protein of mesogenic and velogenic strains. Whereas, trypsin like enzymes that are required to cleave the F0 protein of lentogenic strains can be found in respiratory and intestinal epithelial cells (Nagai, Klenk & Rott 1976).

NDV isolates may be classified into different genotypes based on full fusion protein sequences and the complete genome sequence (Diel et al. 2012). According to the clinical signs produced in infected chickens, strains of NDV have been separated into five groups (Alexander, D 2000b): (I) Viscerotropic velogenic strains causing acute lethal infections, usually causing haemorrhagic lesions in the intestines of dead birds; (II) Neurotropic velogenic strains causing high mortality with neurological disease followed by respiratory sings without gut lesions; (III) Mesogenic strains causing low mortality with respiratory and neurological signs; (IV) Lentogenic strains causing mild infections of the respiratory tract without any sings in the intestinal tract; and (V) Avirulent strains that replicate in the intestine with no clinical signs; these strains are mainly used as live vaccines.

1.3 NDV infection and replication

The virus's attachment to the host cell is mediated by HN protein, and F protein fuses the host cell membrane and the virus (Chang & Dutch 2012). After the virus and the host cell's fusion, the nucleocapsid complex will enter the cell (Senne 2008). A positive-strand mRNA will be produced from the negative strand genome in the cytoplasm by RNA-directed RNA-

polymerase (L protein). The mRNA is translated using host machinery, and M protein transports synthesised viral protein to the modified regions of the cell membrane for assembly before budding (Dortmans et al. 2011). When a sufficient amount of viral proteins have been made, the switch from mRNA to genomic production occurs.

1.4 Host-pathogen interaction

The virus interacts with the host for infection production. NDV evades the host immune system to establish the infection and viral protein. The fusion of NDV to the host cell by HN and F proteins activates complement cascade. Paramyxoviruses include CD46 and CD55 into the viral envelope to inhibit the complement system (Parks & Alexander-Miller 2013). The V protein of NDV blocks interferon signalling by phosphorylating STAT1 to protect the virus against interferon proteins' antiviral effects (Qiu et al. 2016). NDV also incorporates the host proteins into the viral particles to evade the immune system and increase the viral fusion and replication (Ren, X et al. 2012).

On the other side, the host utilises many pathways and genes to combat the virus. NP and N proteins of NDV induce autophagy that may increase viral proteins' presentation on MHC class I (Cheng et al. 2016). Phosphorylated eIF2α by Protein kinase R (PKR) inhibits replication of NDV (Zhang, S et al. 2014). Extracellular matrix molecules like collagen and heparin sulfate limit the spread of NDV (Yaacov et al. 2012).

The innate or cell-mediated immune responses to viral infection aim to help the host to survive. However, it is not always clear that the immune response to velogenic NDV is beneficial or destructive to the host. Chickens are generally unable to survive velogenic NDV without prior vaccination (Miller, Patti J & Koch 2013). Infection caused by velogenic NDV induces a strong iNOS response in the spleen (Rue et al. 2011) and triggered apoptosis of IgM+ cells in the bursa of Fabricius (Kristeen-Teo et al. 2017a). A strong innate immune response does not prevent disease and death caused by velogenic NDV (Rue et al. 2011). While the cellular immune response is critical in viral infection control, the cell-mediated immune response alone cannot provide adequate protection in velogenic NDV infection, and hormonal immunity is also required (Reynolds & Maraqa 2000).

1.5 Vaccination strategy for managing NDV

Lentogenic viruses are non-virulent strain and have been used as vaccine for NDV. La Sota strain is the commonly used vaccine, isolated in New Jersey, the USA in 1946 from Adam La Sota farm (Goldhaft 1980). Vaccination with La Sota strain decreased viral shedding, increased antibody levels and provided 100% protection against virulent CA/2002 virus (Miller, Patti J et al. 2013). If the vaccine and challenge strain are homologous, and enough

time is given to vaccinated birds to develop an immune response, the antibody level can be increased optimally (Yang et al. 2017).

Virus shedding, an improper dosage of vaccination or not vaccinating the entire flock and early disease outbreaks can challenge vaccination program. Roohani *et al.* (2015) also suggested that lentogenic vaccine strains can produce partial protection against velogenic strains (Roohani et al. 2015).

1.6 Newcastle disease in Indonesia and vaccination

Different genotypes of NDV have been circulating worldwide since 1950 (Miller, Patti J., Decanini & Afonso 2010). The first panzootics from the 1920s to the 1960s were caused by genotypes II, III and IV of class II viruses (Alexander, D 2000a), whilst genotype V isolates were responsible for the second epidemic in Europe and Middle-East during the late 1960s (Lomniczi et al. 1998). Genotype *VII.1.1* (b, d, e, j, l) and *VII.2* (a, h, i, k), caused ND panzootic in Africa, Europe, Middle East and Asia (Dimitrov et al. 2019a; Hemmatzadeh 2017; Xiao, Sa, Paldurai, Anandan, et al. 2012).

Recent investigations into the phylogenetic analysis of the F and HN genes of NDV isolates have revealed that most of the NDV isolates from Indonesia's clinical cases belong to genotype *VII* in class *II* (Dharmayanti et al. 2014; Xiao, Sa, Nayak, Baibaswata, et al. 2012). Despite the poultry industry's reliance on vaccination to control the ND since the 1950s, ND still represents a major limiting factor for increasing poultry production in this country (Adi et al. 2010; Dimitrov et al. 2016). Poultry farms are categorised into four sectors according to the flock size and level of the biosecurity. Farms in sector 1 and 2 have acceptable biosecurity practices and are considered independent of the government; thus, the national and regional veterinary services only look after sector 4 and some sector 3 farms.

Although NDV vaccination has been widely practised in all Indonesia sectors, NDV vaccination's efficiency remains unknown because of frequent outbreaks (mainly in sectors 1, 2 and 3). Evidence shows that while there is an overflow of Newcastle disease, sectors 1, 2, and some of sector 3 are more observant to poultry disease issues. These poultry farms work together with private companies to produce new vaccines from viruses isolated from recent outbreaks. The most significant impact of NDV in Indonesia has been estimated to occur in sector 4 production, including village or backyard chickens. NDV outbreaks are the main reason for losses in poultry production in different parts of Indonesia. The most common control practices for NDV is vaccination and/or maintaining strict biosecurity measures. Indonesia's government has introduced new biosecurity standards and strengthened veterinary services and cross-sectional surveillance to improve control of

infectious diseases in poultry. However, vaccination is still considered the best possible long-term solution for control of ND in Indonesia.

According to the Indonesian Veterinary Drugs Index 28 vaccine companies, mostly foreign companies, distribute 246 types of ND vaccines in Indonesia, and they use various ND viruses as their vaccine source. Figure 1.1 shows the type of ND vaccine's seed used by companies in Indonesia(Hemmatzadeh 2017).

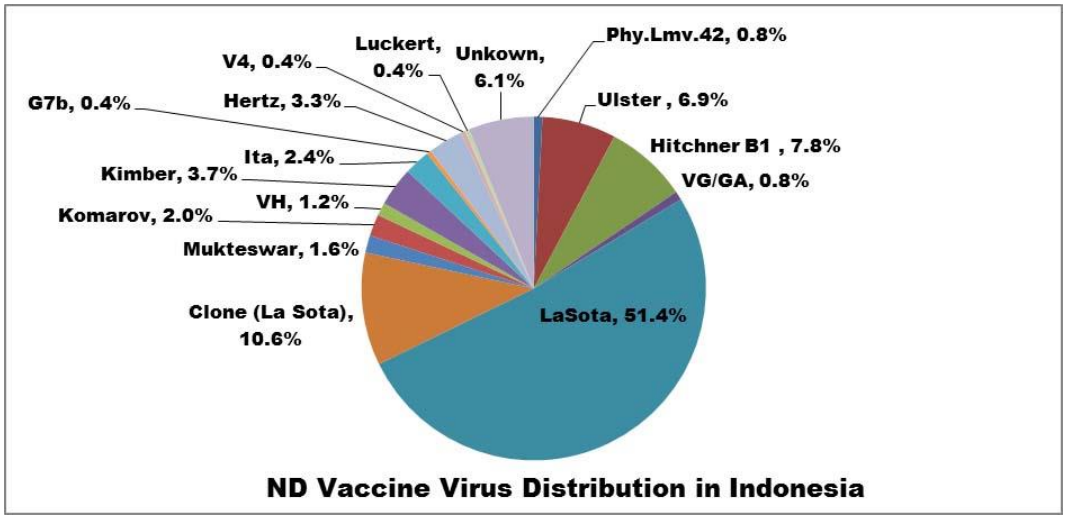

Figure 1.1 ND Vaccine virus distribution in Indonesia.

(Source; Indonesian veterinary drugs index)

Most of these companies use La Sota strain, Clone 30 (or La Sota clone), Hitchner B1, and Ulster as their ND vaccine seeds. There are only 3 Newcastle Disease vaccine distributors from Indonesia that use Ita and G7b local strains from Indonesia as their Newcastle Disease vaccine seed. The level of NDV specific antibodies and the degree of immunity in birds can be assessed by HIT as the method of choice (Balla et al. 1976). Therefore, HIT is frequently used to assess protective immunity after vaccination or as a follow-up test after natural infection in vaccinated flocks. There is a lack of evidence indicating that heterologous NDV antigens show different HI titres in the same serum samples. There may be significant differences between homologous and heterologous NDV antigens in the induction of protective immunity in vaccinated flocks that need to be assessed (Miers, Bankowski & Zee 1983; Miller, Patti J et al. 2007).

The sequence identity matrices of F and HN proteins in Indonesian isolates compare to B1 and La Sota vaccine strains showed 87% to 89% similarity in their amino acid components. This significant difference between vaccine strains and circulating viruses reveals that the circulating strains were substantially different from Indonesia's vaccine strains. These

antigenic differences can explain inefficient vaccine protection in the outbreaks caused by G7 strains (Xiao, S. et al. 2012)

ND is endemic to Indonesia. In 2007, 1,500 to 8,000 cases of ND had been monthly reported by OIE in 2007 (Adi et al. 2010). Since first discovery of NDV in Indonesia in 1926 (Doyle 1927), The poultry industry of this country has been heavily affected by NDV. Recently emerged NDV-GVII in Indonesia poses a threat to neighbouring countries including Australia. Full genome sequencing and phylogenetic analysis and investigation of pathogenesis and tissue tropism of recently emerged NDV in Indonesia helps identify the exact circulating strains and evaluate vaccination strategies' effectiveness, not only in Indonesia but also worldwide because migratory wild birds are considered as possible carriers (Hubálek 2004)Newcastle disease in Australia. Recently emerged NDV started from Indonesia in 2015 and now have been reporting from the rest of Asia, Africa and South America. It has also been considered a significant biosecurity risk, and sporadic outbreak in ND free countries significantly impacts profitability poultry business.

Australia's first cases of virulent ND occurred in 1930 and 1932 (Alkiston & Gorrie 1942; Gould et al. 2001), and both were eradicated by combination of quarantine and slaughter-out policy. Later in mid-1960's, Australian endemic avirulent strains of NDV, was recognized as Queensland V4 strain (Alkiston & Gorrie 1942). Australia is currently free from virulent strains of NDV. However, the introduction of virulent NDV strains is a severe risk for the Australian poultry industry, one of its major industries. ND might occur through spillover from migratory birds (Hubálek 2004). Indonesia is the closest country to Australia and has the second-largest poultry industry in Asia after China (Adi et al. 2010). in the case of spillover of NDV from Indonesia to Australia, it will almost certainly have substantial economic consequences resulting from high death losses of poultry and other birds, mass culling, and international embargos. Our $6.6 billion poultry industry could collapse, resulting in prolonged poultry shortages (Australia's favourite meat) and egg products will follow by jobless people and social disaster (Wilkinson et al. 2014).

1.7 Molecular pathogenesis of NDV

1.7.1 Value of gene expression analysis

The study of the transcriptome has been effectively used in RNA biology. RNA-sequencing (RNA-Seq) analyse all of the mRNAs within a tissue or even a single cell and revolutionised the study of RNA. There is no longer any need to create primers or specific probes for analysing the gene expression level. The high-throughput capability of RNA-Seq can sequence the transcriptome of hundreds of individuals at once. Advanced applications of RNA-Seq such as relative expression, differential expression, RNA editing, alternative

splicing, allele-specific expression, transcript and isoform discovery and gene fusion discovery, made this technology common (Han et al. 2015). RNA-Seq provided the researcher with a more straightforward analysis of gene expression than protein analysis and made gene expression analysis common in biology.

Here, the basic steps of an RNA-Seq experiment are summarised. Extraction of a high-quality RNA is the critical for RNA-Seq. Extracted RNA will be DNase treated to remove any genomic DNA and will be converted into cDNA libraries. The sequencing platform and kit indicates the preparation of libraries. Different sequencing machines have different error rates, run times, yields and cost (Han et al. 2015). The Illumina protocol purifies and fragments the mRNA, syntheses cDNA, and ligates the adaptors to multiplex and uses PCR amplification. Validated libraries will normalise for concentration and will pool and add to a lane within the flow-cell. Adaptors then bind to oligos on the flow cell, and then amplification forms the clusters of double-strand cDNA (Bullard et al. 2010). Nucleotides with fluorescence label will be added in cycles in the sequencing machine, and fluorescent signal records the added nucleotide. The number of cycles indicates the length of the sequence read. The raw reads must go through several steps as quality filtering, mapping the genome and genomic feature count of reads. There are several program and packages available for each of these steps (Han et al. 2015). The edgeR package has been used in this thesis. The number of sequenced reads that map to the gene determines the expression level of that gene.

1.7.2 The impacts of NDV on host gene expression

The impact of NDV on gene expression has been examined in multiple tissues. The qPCR technique has been used to analyse the mRNA expression of key immune genes. Li *et al.* 2016 study showed upregulation of *TLR3/7/21, MDA5, IL2, IL6, IL1β, IFNβ, CXCLi1, CXCLi2, and CCR5* genes in the magnum and uterus of egg-laying hens challenged with velogenic NDV (Li, R et al. 2016). Upregulation of *CCLi3, CXCLi1, CXCLi2, IFNγ, IL12α, IL18, IL1β, IL6, iNOS, TLR7, MHCI, IL17F, and TNFSF13B* genes also measured by qPCR in the spleen of challenged birds with velogenic NDV (Rasoli et al. 2014). Mucosal immunity is heavily involved in the host response to ND infection. Trachea, Harderian glands and lung are places for early virus/host contact at points of entry. These tissues are strategic sites to examine host-pathogen interaction and early viral shedding. Several studies investigated the transcriptome of these tissues infected by NDV (Deist, Gallardo, Bunn, Dekkers, et al. 2017; Deist, Gallardo, et al. 2017a; Deist et al. 2018). In order to increase understanding of the immune response to NDV, the gene expression profile of other immune organs should also be considered.. Recent *in vivo* studies revealed differential regulation of immune response to the lentogenic strain of NDV (La Sota) by transcriptome analysis in the spleen (Zhang, J et

al. 2018; Zhang, J et al. 2020). Another *in vitro* study compared the transcript profile of highly virulent *Herts/33* strain and nonvirulent La Sota strain in spleen cells (Liu, W et al. 2018). The transcriptomic analysis of infection caused by newly emerged virulent NDV-GVII has not been investigated in previous studies.

1.7.3 Apoptosis and NDV

Cell death has been divided into three categories: (1) type I cell death or apoptosis; (2) type II cell death or necrosis; (3) type III cell death or autophagy (Galluzzi et al. 2018). Apoptosis is critical in both physiological and pathological conditions and is known as a multi-pathway process, leading to programmed cell death. This process relies on the activation of different enzymes called caspases, which are proteolytic enzymes that can denature cytoplasmic proteins and cause the nucleus to fragment. Production of caspase-3 known as effector caspase can be considered as a marker of event that apoptosis is on the point (Robbins & Cotran 2009). Apoptosis is a hallmark of cytotoxicity in virus-infected cells with NDV strains that can trigger extrinsic and intrinsic apoptotic pathways (Cuadrado-Castano et al. 2015), and numerous *in-vitro* and *in-vivo* studies have shown that NDV can trigger the apoptosis process (Kalid et al. 2010; Kommers, G et al. 2002; Ravindra et al. 2008; Robbins & Cotran 2009). Different studies have shown that infection with the virulent strains of NDV will increase the apoptosis in lymphoid tissue and immune cells (Brown, King & Seal 1999; Kommers, G et al. 2002; Kommers, GD et al. 2003; Wakamatsu, King, Kapczynski, et al. 2006). Severe splenic disruption, massive lymphoid depletion, ulceration of the intestinal epithelium and rapid depletion of the bursa of Fabricius have been described in association with these strains (Brown, King & Seal 1999; Kommers, G et al. 2002; Kommers, GD et al. 2001, 2003; Wakamatsu, King, Kapczynski, et al. 2006). Other members of *Paramyxoviridae* family such as *Rinderpest* (Stolte et al. 2002), canine distemper, measles (Vidalain et al. 2001) (McCullough, Krakowka & Koestner 1974; Schobesberger et al. 2005), and *porcine Rubulavirus* (Rodríguez-Ropón et al. 2003) similarly targeting the host lymphoid tissues. An important difference between apoptosis and necrosis is that apoptosis does not incite inflammation (Robbins & Cotran 2009).

1.7.4 Necroptosis and NDV

Scientists had been considered necrosis as a rapid, passive and unregulated death process produced by pathological agent as they couldn't associate it with any fixed signal transduction pathway. This was probably the reason that researchers didn't take in to account the necroptosis as an indicator in pathogenesis and treatment of diseases. However, recently revealed findings has challenged the conventional definition of necrosis as purely unregulated cell death process and sheds more lights on deep understanding of

cell death (Galluzzi et al. 2012). Necroptosis or programmed necrosis is the process that necrosis of certain cells is triggered by specific signals and is subject to adjustment of strictly programmed process (Galluzzi & Kroemer 2008). The cytopathic effect of necrotic cell death involves with different sings like karyolysis with mild chromatin condensation, oncosis, loss of integrity of cytoplasmic membrane and cellular swelling. Release of cytoplasmic contents of death cells to extracellular space following massive disintegration of the cell linked to the necrosis results in crucial pro-inflammatory consequences. The severity of Newcastle disease is closely correlated with extensive necrosis. Especially in infections caused by neurotropic and viscerotropic velogenic strains (Wakamatsu, King, Seal, et al. 2006). Velogenic and mesogenic strains of NDV have the capability to induce syncytia that leads to necrosis. After infection of host cells with NDV virus, newly synthesised F and HN protein and the host cells surface accumulate will result in fusion of cell-to-cell membrane (Zeng, Fournier & Schirrmacher 2004). The plasma membrane supports this mechanical stress ending with the syncytium disintegration, and cytoplasmic contents of cells will release in inflammation. Different cellular death events can activate necrotic cell death as a regulated process: such as necroptosis (RIP kinases dependent), parthanatos (PARP dependent) pathways or pyroptosis (inflammasime-dependent) (Pasparakis & Vandenabeele 2015).

Recent research highlighted necroptosis as a part of the molecular pathway activated in infected glioblastoma cells with NDV (Koks et al. 2015). Necroptosis can be activated as an alternative form of programmed cell death by different molecular triggers like DNA and RNA sensors, TNF members and Toll-like receptors. This happens in a caspase 8 independent manner, and the signal activity depends on receptor interacting protein kinase 1(RIPK1)-RIPK3 complex. Koks *et al* study (2015) also confirmed that Necrostatin-1 inhibits this signal (Kaczmarek, Vandenabeele & Krysko 2013). They also mentioned that Hitchner B1 strain of NDV caused necrosis-like morphologic changes like karyolysis and cellular swelling in GL2I61 glioma cells that were infected with this strain of NDV. This is the first report of programmed necrosis as a result of experimentally infection by NDV highlighting the molecular characterisation of cell death. This study showed dispensability activated by caspase which is sensitive to Necrostatin-1 and significantly rescued the cytotoxicity caused by virulent strain of NDV. There is also an evidence of increased population of Annexin-V(-)/PI(+) cells during the time course of infection by NDV.

1.7.5 DNA damage and NDV

Viruses are obligate intracellular parasites and use the host cell transcriptome machinery to replicate viral genome and production of viral protein. This virus-host cell interaction involves DNA transactions, including the induction of DNA damage. Viruses have to deal with the

host DNA damage machinery, as host cells limit the DNA damage to constrain and localise the viruses. The host cell uses DNA damage response (DDR) signalling to induce cell cycle arrest to mitigate damage induction, promote repair, or even induce cell death. Significant DNA damage levels can lead DDR proteins to start apoptotic programmed cell death to preserve host genomic integrity (Luftig 2014).

The Innate immune system plays a crucial role in the immune response to viral infection, and oxidative stress has a crucial impact on the innate immune system. Production of reactive oxygen species (ROS) and prooxidant cytokines during activation of innate immune cells in viral infection enhances the mononuclear phagocytic system (reticuloendothelial system) (Schwarz 1996). Viral infection increases oxidants' production, such as superoxide anion (O_2^-) and nitric oxide (NO). It prevents the synthesis of antioxidant enzymes, such as catalases (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Reshi, Su & Hong 2014). Immune cells need high quantities of antioxidant enzymes compared to other cells, and the limited production and activity of these enzymes lead to a weakened immune response. Production of ROS during viral infections from granulocytes and macrophages exerts antimicrobial action against many pathogens (Fang 2011). ROS also trigger other pathways to kill or spread viral infections, including autophagy (Huang, J, Lam & Brumell 2011) and apoptosis (Skulachev 1998). Virus-induced oxidative stress also causes DNA damage by modifying the nucleobases and sugar backbone and results in strand breakages, crosslinking and base loss (Cadet & Davies 2017). If unrepaired, these lesions can be mutagenic and compromise genome integrity.

Most current studies have focused on the interface between the cellular DDR pathway and DNA virus infection (Schmid et al. 2014). However, only a few studies have investigated the RNA viruses and DDR pathway (Ryan, Hollingworth & Grand 2016). No investigation of DNA damage levels or DDR signalling has occurred in chickens infected with the newly emerged NDV-GVII.

1.8 Thesis objectives

This thesis characterises the full genome sequence of two strains caused outbreaks in Indonesia. The molecular basis of pathogenesis of newly emerged NDV-GVII will be identified using mRNA profiling of spleen tissues in experimentally infected chickens. The tissue tropism and DNA damage associated with oxidative stress, apoptosis and necroptosis patterns in different tissues of experimentally infected chickens with virulent strains of NDV will also be evaluated. These results will enable the identification of genes and pathways related to newly emerged NDV. Additionally, this project's result will shed light on the tissue tropism and pathology of massive lymphoid depletion caused by this strain.

2 Genome sequences of newly emerged Newcastle disease virus strains isolated from disease outbreaks in Indonesia

2.1 Statement of authorship

Title of Paper	Genome Sequences of newly emerged Newcastle disease virus strains isolated from disease outbreaks in Indonesia		
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style		
Publication Details	Microbiology Resource Announcements; 2020; 9:e00204-20. DOI: 10.1128/MRA.00204-20		
Name of Principal Author (Candidate)	Mohammad Rabiei		
Contribution to the Paper	Developed the hypothesis and designed the experimental work, isolated RNA, constructed cDNA libraries, processed and analysed data, wrote the paper.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	02/02/2020
Name of Co-Author	Mohamad Indro Cahyono		
Contribution to the Paper	Participated in the design and sampling, and was involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	04/03/2021
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Contribution to the Paper	Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	04/02/2021
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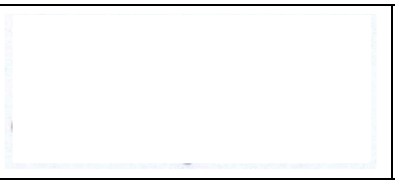
Signature		Date	08/02/2021
Name of Co-Author	Wai Yee Low		
Contribution to the Paper	Provided advice on the bioinformatics, I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	10/02/2021
Name of Co-Author	Rick Tearle		
Contribution to the Paper	Provided advice on the bioinformatics. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
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Signature		Date	03/02/2021

Name of Co-Author	Mohammed Alsharifi		
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Signature		Date	09/02/2021
Name of Co-Author	Farhid Hemmatzadeh		
Contribution to the Paper	Participated in the design of study, supervised the project and Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	03/02/2021

Genome Sequences of newly emerged Newcastle disease virus strains isolated from disease outbreaks in Indonesia.

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2.2 Abstract

Here, we report two genomes of virulent strains of *Newcastle disease virus (NDV) Tangerang/004WJ/14 and VD/003WJ/11*, from disease outbreaks in chickens in Indonesia. Phylogenetic study results of Fusion (F) protein's gene coding sequences of different genotypes of NDV revealed that these two strains belong to genotype *VII.1*, class II cluster of *avian paramyxoviruses*.

2.3 Keywords

Newcastle disease virus, Genome Sequences, NDV vaccine, next-generation sequencing

2.4 Introduction

Newcastle disease (ND) still causes considerable mortality and reduces profitability in South-East Asia's chicken industry and as an endemic disease in Indonesia. Newcastle disease virus (NDV), is a member of the genus Avian *orthoavulavirus1* within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* (Rima et al. 2019a). Genotype *VII.1.1* (b, d, e, j, l) and *VII.2* (a, h, i, k), caused ND panzootic in Africa, Europe, Middle East and Asia (Dimitrov et al. 2019a; Hemmatzadeh 2017; Xiao, Sa, Paldurai, Anandan, et al. 2012). This study

compared full-length genomes of virulent strains strains of VII NDVs to currently used La Sota vaccine strain.

2.5 Materials and methods

Chicken/Indonesia/Tangerang/004WJ/14 and *Chicken/Indonesia/VD/003WJ/11* strains were isolated from brain samples of two NDV vaccinated chicken with the live La Sota vaccine. The viruses were isolated from two-layer farms with high mortality in different geographical locations in West Java, Indonesia in 2011 and 2014. According to OIE standard protocol, the viruses were isolated by inoculation of embryonated Specific Pathogen Free (SPF) chicken eggs and harvest of allantoic fluid (Stear 2005). According to the FAO manual, the pathogenicity of these strains was measured by Mean Death Time (MDT) assay (Grimes 2002) at the Indonesian Research Center for Veterinary Sciences (Bbalitvet). A commercial Viral RNA Mini Kit (QIAamp Viral RNA Mini Kit, QIAGEN, USA) was used for RNA extraction. The extracted RNA was submitted to ACRF (Australian Cancer Research Foundation) for RNA sequencing. The cDNA library and sequencing were done by ACRF and used a random hexamer approach (KAPA Stranded mRNA-Seq Kit, KAPA Biosystems, USA) manufacturer's recommendations. The Illumina MiSeq platform v3 was used for sequencing cDNA libraries and generated 2x300 nt reads. After removing adaptors and low quality reads by Trimmomatic 0.36 software (Bolger, Lohse & Usadel 2014), the Unicycler v.0.4.4 software was used for de novo assembly of 817,686 reads for sample 1 764,502 reads for sample 2. Final assembled reads were visualised by Bandage (Wick et al. 2015). Contigs were compared to nucleotide collection using NCBI BLAST. Two NDV contigs for Tangerang and VD strains were identified with 46.52% and 46.49% genome GC content, 15096 and 15179 nucleotide length and 818-fold and 534-fold coverage respectively. These contigs were compared to the Indonesian genotype *VII* strain of *chicken/Sukorejo/019/10* (GenBank accession no. HQ697255.1) and showed a similarity of 97.90% and 98.95%. In the contig of Tangerang strain some contaminating sequences of *Pseudomonas* were observed after NCBI BLAST at the end of the sequence which was removed by BioEdit and RT-PCR (QIAGEN OneStep Ahead RT-PCR, QIAGEN, USA) and Sanger sequencing were used to close detected gaps after aligned to *Sukorejo* (GenBank accession no. HQ697255.1) in this sequence. Clustal X(Larkin et al. 2007) and Genious Primer software(Kearse et al. 2012) programs were used to align and annotate genes. All tools were run with default parameters.

2.6 Results

Both *Tangerang/004WJ/14* and *VD/003WJ/11*strains are associated with severe neurological sings in infected chickens and had an MDT of 52 and 33 hours. These two strains are similar at the C terminal of the F protein cleavage site, a key molecular marker for NDV pathogenicity (de Leeuw et al. 2005; Panda et al. 2004). The ^{111}RRRKR↓F^{117} amino acid sequence motif is the same as *chicken/Sukorejo/019/10* as a reference strain for Indonesian genotype VII of NDVs. Phylogenetic analysis of F gene sequences carried out using Mega 7 software(Kumar, Stecher & Tamura 2016), suggests that these circulating strains in Indonesia belong to genotype VII.1 (Figure 2.1), as the main genotype causing recent NDV outbreaks in Indonesia. Notably, the amino acid sequence for viral N, P, M, F, HN and L proteins for these two strains were similar and have percentage identities of 92%, 81%, 88%, 89%, 85% and 94%, to the *La Sota* strain (GenBank accession no. AF077761.1) respectively.

2.7 Discussion

These significant differences in amino acid identities of circulation viruses and *La Sota* strain as the most common vaccine strain used in Indonesia shed more light on the probable reason for vaccine failure against these NDV strains and highlight the urgent need for updated vaccine development strategies in South-East Asia.

2.8 Data availability

The GenBank accession numbers for *Tangerang/004WJ/14* and *VD/003WJ/11* are MN699677 and MN699676, respectively. The BioSample SRA run numbers are SRR11593162 for Tangerang and SRR11593166 for VD.

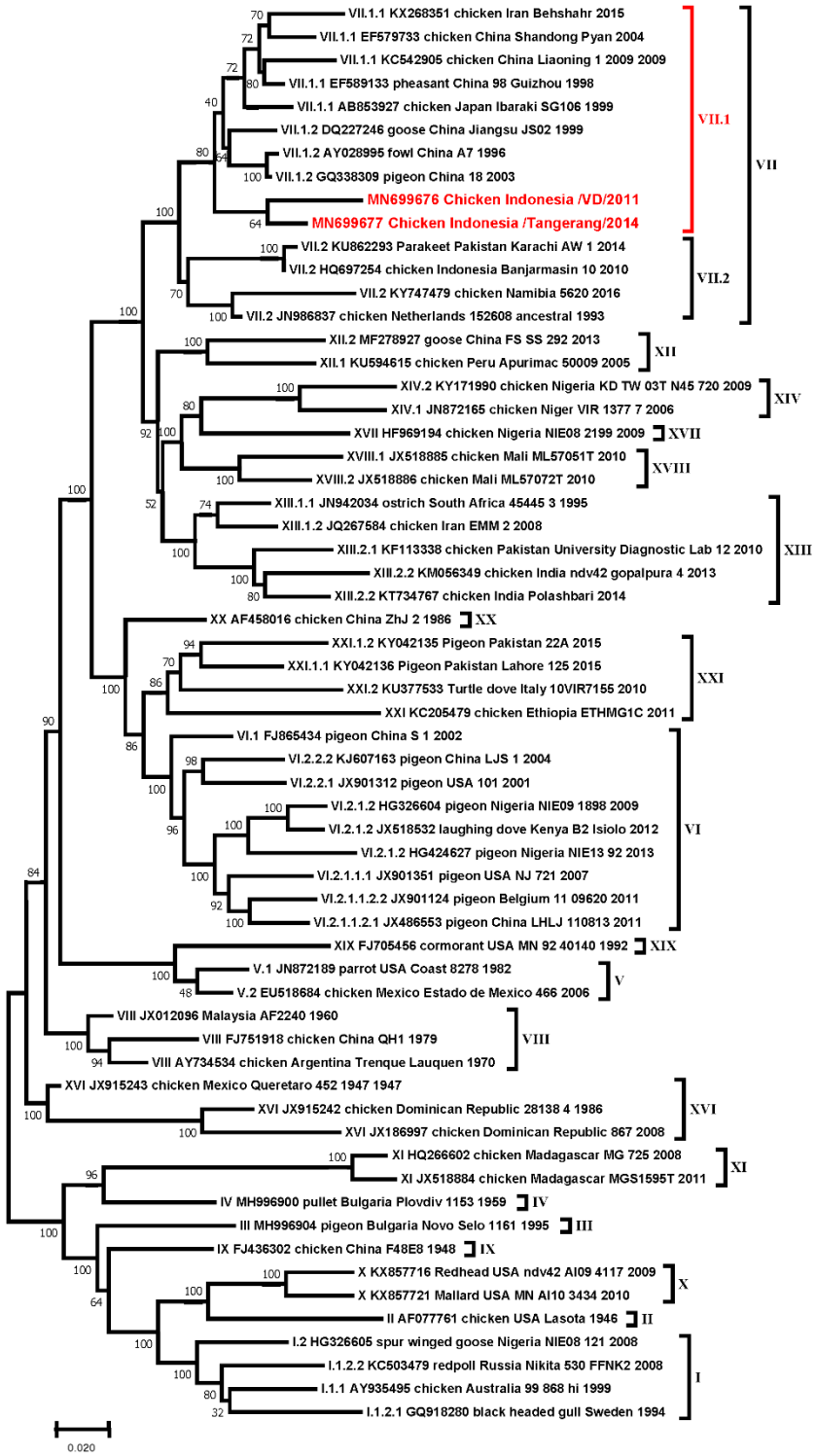

Figure 2.1 Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei 1993). The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein 1985). Initial tree(s) for the heuristic search were obtained

automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The analysis involved 70 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 326 positions in the final dataset. Evolutionary analyses were conducted in MEGA7(Kumar, Stecher & Tamura 2016). Two strains of subgenotype VII.2 highlighted in red are from this study.

2.9 Acknowledgements

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2.10 Reprint of published paper

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GENOME SEQUENCES

Genome Sequences of Newly Emerged Newcastle Disease Virus Strains Isolated from Disease Outbreaks in Indonesia

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ABSTRACT Here, we report two genomes of newly emerged strains of *Newcastle disease virus* (NDV), Chicken/Indonesia/Tangerang/004WJ/14 and Chicken/Indonesia/VD/003WJ/11, from disease outbreaks in chickens in Indonesia. Phylogenetic study results of the fusion (F) protein's gene-coding sequences of different genotypes of NDV revealed that these two strains belong to genotype VII.2 in the class II cluster of avian paramyxoviruses.

Newcastle disease (ND) still causes high mortality and reduces profitability in the chicken industry in Southeast Asia and is an endemic disease in Indonesia. Newcastle disease virus (NDV) is a member of the genus *Avian orthoavulavirus 1* within the new subfamily *Avulavirinae* of the family *Paramyxoviridae* (1). Genotypes VII.1.1 (subgenotypes b, d, e, j, and l) and VII.2 (subgenotypes a, h, i, and k) caused an ND panzootic in Africa, Europe, the Middle East, and Asia (2–4). In this study, we compared the full-length genomes of newly emerged strains of genotype VII NDVs to that of the currently used vaccine strain, LaSota.

Strains Chicken/Indonesia/Tangerang/004WJ/14 (Tangerang) and Chicken/Indonesia/VD/003WJ/11 (VD) were isolated from the brain samples of two chickens vaccinated against NDV with the live LaSota vaccine. The viruses were isolated from two layer farms with high mortality located in different geographical locations in West Java, Indonesia, in 2011 and 2014. The viruses were isolated by inoculating embryonated specific-pathogen-free (SPF) chicken eggs and harvesting allantoic fluid according to World Organisation for Animal Health (OIE) standard protocol (5). The pathogenicity of these strains was measured by mean death time (MDT) assay according to the Food and Agriculture Organization (FAO) manual (6). A QIAamp viral RNA minikit (Qiagen, USA) was used for RNA extraction, and the extracted RNA was submitted to the Australian Cancer Research Foundation (ACRF) for RNA sequencing. The cDNA library and sequencing were performed by the ACRF using a random hexamer approach (stranded mRNA-Seq kit; Kapa Biosystems, USA) as per the manufacturer's recommendations. The Illumina MiSeq platform v3 was used for sequencing cDNA libraries and generated 2 × 300-nucleotide (nt) reads. After removing the adaptors and low-quality reads with Trimmomatic v0.36 software (7), Unicycler v.0.4.4 software was used for *de novo* assembly of a total of 817,686 reads for sample 1 and 764,502 reads for sample 2. The final assembled reads were visualized using Bandage (8). Contigs were compared to the

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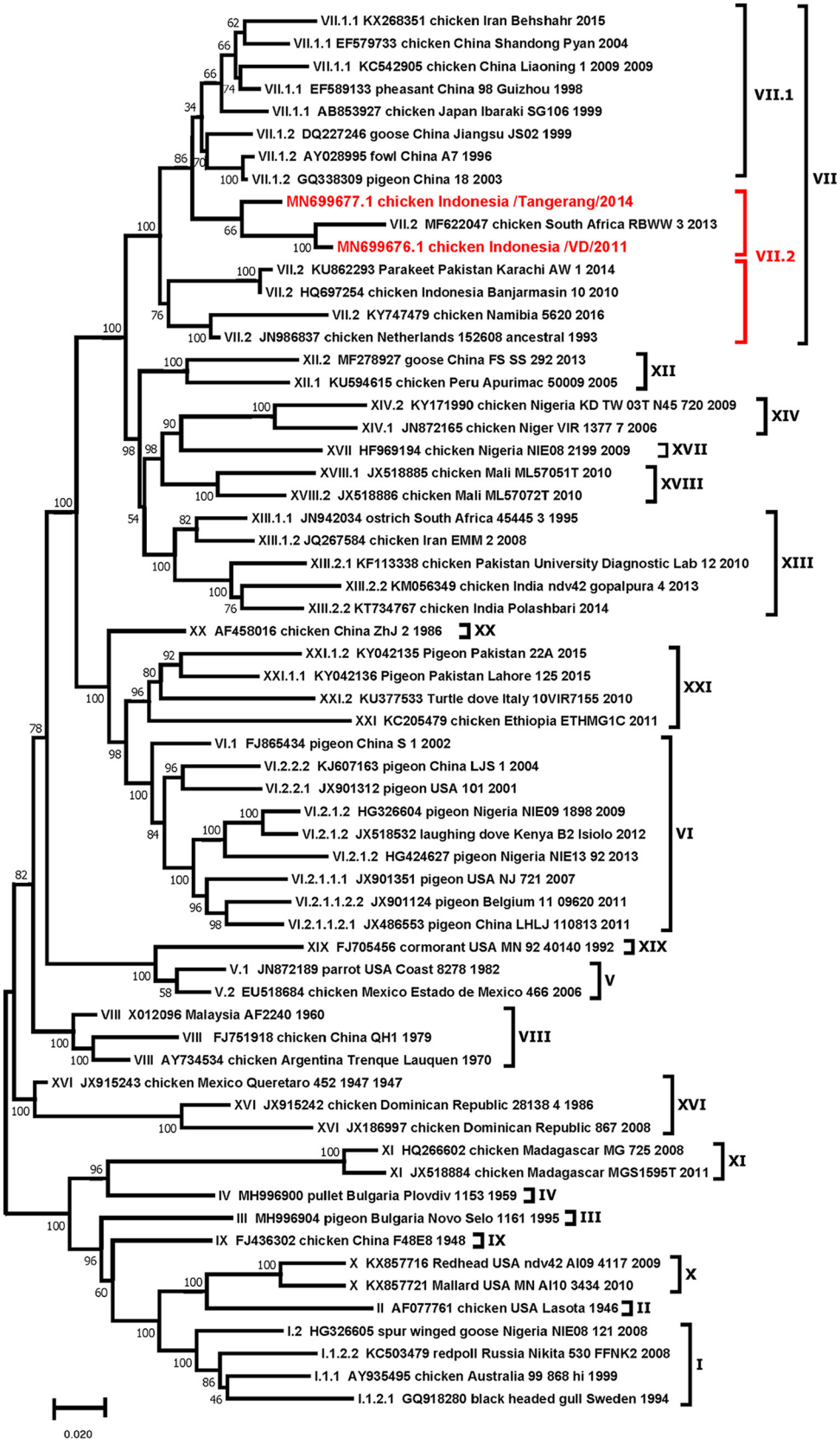

FIG 1 Molecular phylogenetic analysis by the maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (14). The bootstrap consensus tree

(Continued on next page)

FIG 1 Legend (Continued)
inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed (15). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (15). The initial tree(s) for the heuristic search was obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The analysis involved 70 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 326 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (13). The two strains of subgenotype VII.2 highlighted in red are from this study.

nucleotide collection using NCBI BLAST, and two NDV contigs for Tangerang and VD were identified, with 46.52% and 46.49% genome GC contents, 15,096 and 15,179 nucleotide lengths, and 818-fold and 534-fold coverages, respectively. These contigs were compared to the Indonesian genotype VII strain Chicken/Sukorejo/019/10 (Sukorejo; GenBank accession number HQ697255.1) and showed similarities of 97.90% and 98.95%, respectively. In the contig of Tangerang, some contaminating sequences of *Pseudomonas* spp. were observed after an NCBI BLAST search at the end of the sequence; they were removed with BioEdit, and reverse transcriptase PCR (RT-PCR; OneStep Ahead RT-PCR, Qiagen, USA) and Sanger sequencing were used to close the detected gaps in this sequence after alignment to Sukorejo (GenBank accession number HQ697255.1). The Clustal X (9) and Genious Primer (10) software programs were used to align and annotate genes. All tools were run with default parameters.

Both Tangerang and VD are associated with severe neurological symptoms in infected chickens and had a mean death time (MDT) of 60 to 67 h. These two strains are similar at the C terminus of the F protein cleavage site, which is a key molecular marker for NDV pathogenicity (11, 12). The ^{111}RRRKR ↓ F^{117} amino acid sequence motif is the same as that in Sukorejo, as the reference strain for the Indonesian genotype VII of NDVs. Phylogenetic analysis of F gene sequences carried out using MEGA7 software (13) suggests that these strains circulating in Indonesia belong to genotype VII.2 (Fig. 1), the main genotype causing recent NDV outbreaks in Indonesia. Importantly, the amino acid sequences for the viral N, P, M, F, HN, and L proteins for these two strains were similar and have percent identities of 92%, 81%, 88%, 89%, 85%, and 94% to strain LaSota (GenBank accession number AF077761.1), respectively.

These significant differences in the amino acid identities of circulation viruses and strain LaSota, as the most common vaccine strain used in Indonesia, shed more light on the probable reason for vaccine failure against these NDV strains and highlight the urgent need for updated vaccine development strategies in Southeast Asia.

Data availability. The GenBank accession numbers for Tangerang and VD are MN699677 and MN699676, respectively. The BioSample SRA run numbers are SRR11593162 for Tangerang and SRR11593166 for VD.

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AUTHOR CORRECTION

Correction for Rabiei et al., "Genome Sequences of Newly Emerged Newcastle Disease Virus Strains Isolated from Disease Outbreaks in Indonesia"

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Volume 9, no. 23, e00204-20, 2020, https://doi.org/10.1128/MRA.00204-20. Page 1, abstract, line 5: "genotype VII.2" should read "genotype VII.1."

Page 2: Figure 1 should appear as shown in this correction.

Page 3, figure legend, lines 8 and 9: "subgenotype VII.2" should read "subgenotype VII.1."

Page 3, line 19: "genotype VII.2" should read "genotype VII.1."

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Risa Indriani was unreachable for approval of this correction.

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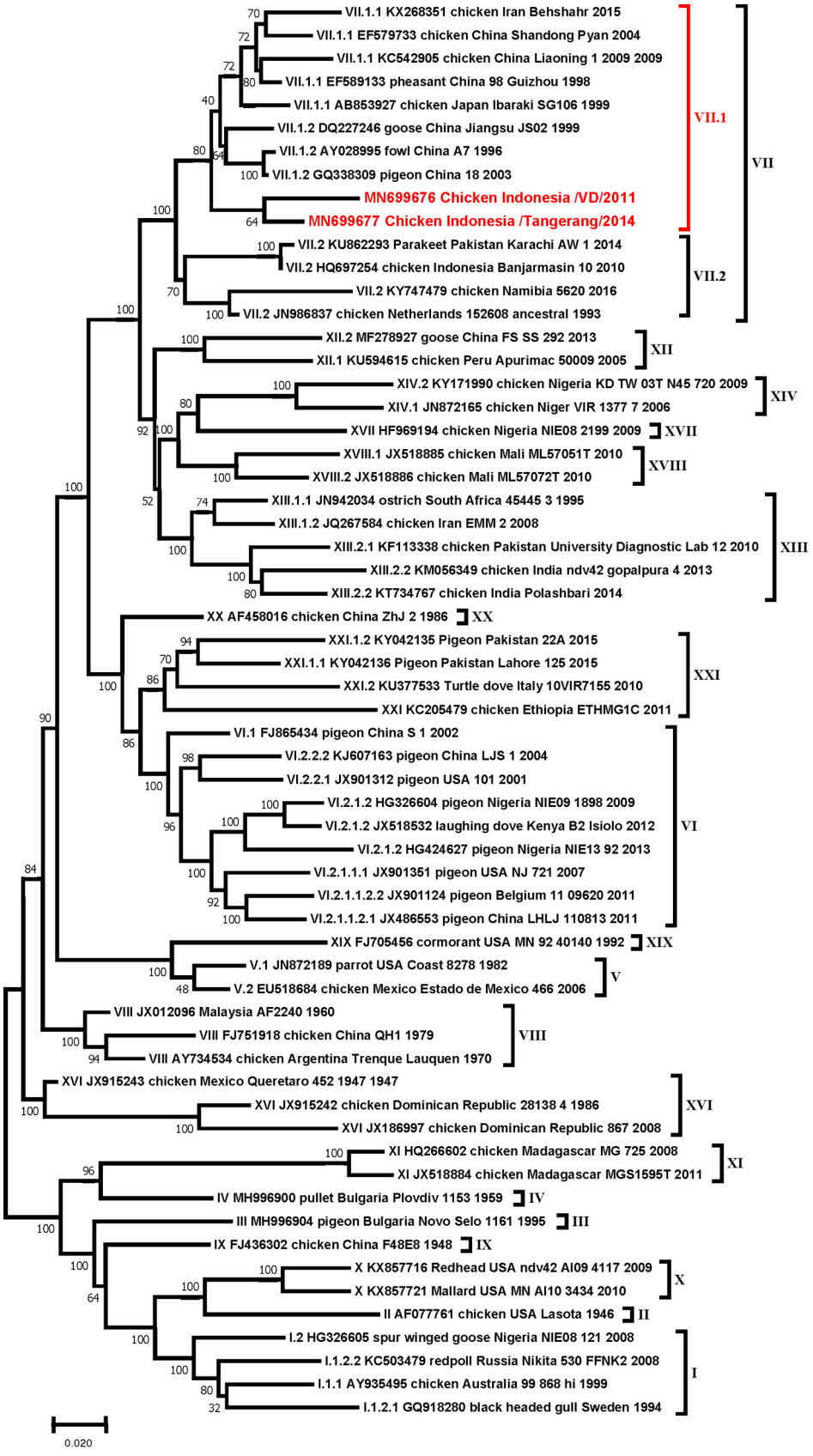
VII.1.1 KX268351 chicken Iran Behshahr 2015
VII.1.1 EF579733 chicken China Shandong Pyan 2004
VII.1.1 KC542905 chicken China Liaoning 1 2009 2009
VII.1.1 EF589133 pheasant China 98 Guizhou 1998
VII.1.1 AB853927 chicken Japan Ibaraki SG106 1999
VII.1.2 DQ227246 goose China Jiangsu JS02 1999
VII.1.2 AY028995 fowl China A7 1996
VII.1.2 GQ338309 pigeon China 18 2003
MN699676 Chicken Indonesia /VD/2011
MN699677 Chicken Indonesia /Tangerang/2014
VII.1
VII.2 KU862293 Parakeet Pakistan Karachi AW 1 2014
VII.2 HQ697254 chicken Indonesia Banjarmasin 10 2010
VII.2 KY747479 chicken Namibia 5620 2016
VII.2 JN986837 chicken Netherlands 152608 ancestral 1993
VII.2
VII
XII.2 MF278927 goose China FS SS 292 2013
XII.1 KU594615 chicken Peru Apurimac 50009 2005
XII
XIV.2 KY171990 chicken Nigeria KD TW 03T N45 720 2009
XIV.1 JN872165 chicken Niger VIR 1377 7 2006
XIV
XVII HF969194 chicken Nigeria NIE08 2199 2009
XVII
XVIII.1 JX518885 chicken Mali ML57051T 2010
XVIII.2 JX518886 chicken Mali ML57072T 2010
XVIII
XIII.1.1 JN942034 ostrich South Africa 45445 3 1995
XIII.1.2 JQ267584 chicken Iran EMM 2 2008
XIII.2.1 KF113338 chicken Pakistan University Diagnostic Lab 12 2010
XIII.2.2 KM056349 chicken India ndv42 gopalpura 4 2013
XIII.2.2 KT734767 chicken India Polashbari 2014
XIII
XX AF458016 chicken China ZhJ 2 1986
XX
XXI.1.2 KY042135 Pigeon Pakistan 22A 2015
XXI.1.1 KY042136 Pigeon Pakistan Lahore 125 2015
XXI.2 KU377533 Turtle dove Italy 10VIR7155 2010
XXI KC205479 chicken Ethiopia ETHMG1C 2011
XXI
VI.1 FJ865434 pigeon China S 1 2002
VI.2.2.2 KJ607163 pigeon China LJS 1 2004
VI.2.2.1 JX901312 pigeon USA 101 2001
VI.2.1.2 HG326604 pigeon Nigeria NIE09 1898 2009
VI.2.1.2 JX518532 laughing dove Kenya B2 Isiolo 2012
VI.2.1.2 HG424627 pigeon Nigeria NIE13 92 2013
VI.2.1.1.1 JX901351 pigeon USA NJ 721 2007
VI.2.1.1.2.2 JX901124 pigeon Belgium 11 09620 2011
VI.2.1.1.2.1 JX486553 pigeon China LHLJ 110813 2011
VI
XIX FJ705456 cormorant USA MN 92 40140 1992
XIX
V.1 JN872189 parrot USA Coast 8278 1982
V.2 EU518684 chicken Mexico Estado de Mexico 466 2006
V
VIII JX012096 Malaysia AF2240 1960
VIII FJ751918 chicken China QH1 1979
VIII AY734534 chicken Argentina Trenque Lauquen 1970
VIII
XVI JX915243 chicken Mexico Queretaro 452 1947 1947
XVI JX915242 chicken Dominican Republic 28138 4 1986
XVI JX186997 chicken Dominican Republic 867 2008
XVI
XI HQ266602 chicken Madagascar MG 725 2008
XI JX518884 chicken Madagascar MGS1595T 2011
XI
IV MH996900 pullet Bulgaria Plovdiv 1153 1959
IV
III MH996904 pigeon Bulgaria Novo Selo 1161 1995
III
IX FJ436302 chicken China F48E8 1948
IX
X KX857716 Redhead USA ndv42 AI09 4117 2009
X KX857721 Mallard USA MN AI10 3434 2010
X
II AF077761 chicken USA Lasota 1946
II
I.2 HG326605 spur winged goose Nigeria NIE08 121 2008
I.1.2.2 KC503479 redpoll Russia Nikita 530 FFNK2 2008
I.1.1 AY935495 chicken Australia 99 868 hi 1999
I.1.2.1 GQ918280 black headed gull Sweden 1994
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3 Indicators of the molecular pathogenesis of virulent Newcastle disease virus in chickens revealed by transcriptomic profiling of spleen

3.1 Statement of authorship

Title of Paper	Indicators of the molecular pathogenesis of virulent Newcastle disease virus in chickens revealed by transcriptomic profiling of spleen
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Name of Principal Author (Candidate)	Mohammad Rabiei		
Contribution to the Paper	Developed the hypothesis and designed the experimental work, isolated RNA, constructed cDNA libraries, processed and analysed data, wrote and revised the original paper.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	06/02/2021

Name of Co-Author	Wai Yee Low		
Contribution to the Paper	Provided advice on the bioinformatics. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	10/6/2016

Name of Co-Author	Milton M. McAllister		
Contribution to the Paper	Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	04/02/2021

Name of Co-Author	Yan Ren		
Contribution to the Paper	Provided advice on the bioinformatics. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	10/02/2021
Name of Co-Author	Mohamad Indro Cahyono		
Contribution to the Paper	Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	04/03/2021
Name of Co-Author	Phuong Thi Kim Doan		
Contribution to the Paper	Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	04/02/2021
Name of Co-Author	Indi Dharmayanti		
Contribution to the Paper	Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	04/03/2021
Name of Co-Author	Eleonora Dal Grande		
Contribution to the Paper	Provided advice on the statistics. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	10/02/2021

Name of Co-Author	Farhid Hemmatzadeh		
Contribution to the Paper	Participated in the study design, supervised the project and Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	10/02/2021

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3.3 Abstract

Newcastle disease virus (NDV) has caused significant outbreaks in South-East Asia, particularly in Indonesia. Recently emerged genotype VII NDVs (NDV-GVII) have shifted their tropism from gastrointestinal/respiratory tropism to a lymphotropic virus, invading lymphoid organs, including spleen and Bursa of Fabricius to cause profound lymphoid depletion especially in spleen. This study aimed to identify candidate genes and biological pathways that contribute to the disease caused by this neurotropic velogenic NDV-GVII. A transcriptomic analysis based on RNA-Seq of spleen was performed in chickens challenged with NDV-GVII and a control group inoculated with strile medium. In total, 6361 genes were differentially expressed that included 3506 up-regulated genes and 2855 down-regulated genes. Real-time PCR of ten selected genes validated the RNA-Seq results as the correlation between them is 0.98. Functional and network analysis of differentially expressed genes (DEGs) showed down regulation of EIF2 signalling, mTOR signalling, the proliferation of cells of the lymphoid system, signalling by Rho family GTPases and synaptogenesis signalling in spleen. We have also identified increased expression of *IFIT5, PI3K, AGT* and *PLP1* genes in NDV-GVII infected chickens. Our findings in activation of autophagy-mediated cell death, lymphotropic and synaptogenesis signalling pathways provide new insights into the molecular pathogenesis of this virulent strains NDV-GVII.

3.4 Keywords

Newcastle disease virus, Gene expression profile, Chicken spleen, Apoptosis, Necroptosis, Autophagy

3.5 Introduction

Newcastle disease virus (NDV) has a worldwide distribution. The NDV causes infection in many different avian species, and it can be considered a permanent threat to all poultry industries and other fields of aviculture (Miller, Patti J et al. 2015). NDV is capable of causing devastating infection for over 240 species of birds that can spillover through direct contact between healthy and infected birds (Alexander, DJ, Aldous & Fuller 2012; Miller, Patti J., Decanini & Afonso 2010). NDV was first reported in Java, Indonesia in 1926 and then spread to the rest of the world. Various genotypes have been responsible for different ND panzootics (Doyle 1927). Most recent ND outbreaks in Southeast Asia are mainly caused by highly virulent NDV-GVII leading to 70-80% mortality in commercial chickens, including vaccinated flocks (Adi et al. 2010; Xiao, Sa, Nayak, Baibaswata, et al. 2012). While genotype VII NDVs includes a wide variety of sub-genotypes, the fourth and the fifth ND panzootic in Africa, Europe, Middle East and Asia were caused by genotype VII.1.1 (b, d, e, j, l) and VII.2 (a, h, i, k), respectively (Dimitrov et al. 2019b; Hemmatzadeh 2017; Xiao, Sa, Nayak, Baibaswata, et al. 2012).

NDV is a member of the genus avian *orthoavulavirus 1* within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* (Dimitrov et al. 2019b). NDV has a negative-sense single-stranded, non-segmented RNA genome that encodes six major structural protein genes in the order 3'-NP-P-M-F-HN-L-5' (Rabiei et al. 2020). According to the clinical signs in infected chickens, different strains of NDV have been separated into five groups (Afonso et al. 2012): (I) Viscerotropic velogenic strains causing acute lethal infections, usually causing haemorrhagic lesions in the intestines; (II) Neurotropic velogenic strains causing high mortality with neurological disease followed by respiratory sings without gut lesions; (III) Mesogenic strains causing low mortality with respiratory and neurological signs; (IV) Lentogenic strains causing mild infections of the respiratory tract without any lesions in the intestinal tract; and (V) Avirulent strains that replicate in the intestine with no clinical signs. Avirulent strains are often used as live vaccines (Xiao, Sa, Nayak, Baibaswata, et al. 2012). Phylogenetic analysis of the fusion protein gene of NDVs indicates that circulating strains in Indonesia belong to genotype VII.1 with a mean death time (MDT) from 30 to 33 hour as their pathogenicity index (Doan et al. 2020; Pandarangga et al. 2020; Rabiei et al. 2020). These findings also indicate that these circulating strains are clinically categorised as neurotropic velogenic. In this study, we have used one of these genotype VII NDV strains in our challenge experiment to analyse pathogenesis of this virulent strains NDV (Doan et al. 2020). The amino acid composition of the fusion protein's cleavage site and its susceptibility to host trypsin-like proteases play a big role in pathogenicity, the spread of infection, and tissue tropism of NDVs (Nagai 1995). Mucosal immunity is heavily involved in the host

response to ND infection. Trachea, Harderian glands and lung are places for early virus/host contact at points of entry. These tissues are strategic sites to examine host-pathogen interaction and early viral shedding. Several studies investigated the transcriptome of these tissues infected by NDV (Deist, Gallardo, Bunn, Dekkers, et al. 2017; Deist, Gallardo, et al. 2017a; Deist et al. 2018). In order to increase understanding of the immune response to NDV, the gene expression profile of other immune organs should also be considered. Recent *in vivo* studies revealed differential regulation of immune response to the lentogenic strain of NDV (La Sota) by transcriptome analysis in the spleen (Zhang, J et al. 2018; Zhang, J et al. 2020). Another *in vitro* study compared the transcript profile of highly virulent *Herts/33* strain and nonvirulent La Sota strain in spleen cells (Liu, W et al. 2018). The transcriptomic analysis of infection caused by virulent strains virulent NDV-GVII has not been investigated in previous studies.

Cell death has been divided into three categories: (1) type I cell death or apoptosis; (2) type II cell death or necrosis; (3) type III cell death or autophagy (Galluzzi et al. 2018). Apoptosis is critical in both physiological and pathological conditions and is known as a multi-pathway process, leading to programmed cell death. Apoptosis is associated with many types of viral infections and, depending on the circumstances, can act to increase or decrease viral production. Apoptosis is a hallmark of cytotoxicity in virus-infected cells with NDV strains that can trigger extrinsic and intrinsic apoptotic pathways (Cuadrado-Castano et al. 2015), and numerous *in-vitro* and *in-vivo* studies have shown that NDV can trigger the apoptosis process (Kalid et al. 2010; Kommers, G et al. 2002; Ravindra et al. 2008; Robbins & Cotran 2009). Different studies have shown that infection with the virulent strains of NDV will increase the apoptosis in lymphoid tissue and immune cells (Brown, King & Seal 1999; Kommers, G et al. 2002; Kommers, GD et al. 2003; Wakamatsu, King, Kapczynski, et al. 2006). Severe splenic disruption, massive lymphoid depletion, ulceration of the intestinal epithelium and rapid depletion of the bursa of Fabricius have been described in association with these strains (Brown, King & Seal 1999; Kommers, G et al. 2002; Kommers, GD et al. 2001, 2003; Wakamatsu, King, Kapczynski, et al. 2006). Other members of *Paramyxoviridae* family such as *Rinderpest* (Stolte et al. 2002), canine distemper, measles (Vidalain et al. 2001) (McCullough, Krakowka & Koestner 1974; Schobesberger et al. 2005), and *porcine Rubulavirus* (Rodríguez-Ropón et al. 2003) similarly targeting the host lymphoid tissues. An important difference between apoptosis and necrosis is that apoptosis does not incite inflammation (Robbins & Cotran 2009).

The amino acid sequence motif ^{112}RRQKRF^{117} of fusion protein has been previously indicated as the neuropathogenic genetic markers of virulent NDV (Adi et al. 2010; Hamid, Campbell & Parede 1991; Parede & Young 1990). Our previous studies have discovered

and reported two different sub-genotypes of NDV GVII that contain RRQKRF motif in Mega strain and RRRKRF in Cimanglid and VD strains (Doan et al. 2020; Pandarangga et al. 2020; Rabiei et al. 2020). The Mega strain used in our challenge experiment carries the RRQKRF motif sequence in the fusion protein, and has been reported as neuropathonenicity indicator of the NDV even in genetically modified lentogenic strains (Orsi et al. 2009; Panda et al. 2004). In addition to these references, the Mega strain has been isolated from a brain of a dead chicken (Doan et al. 2020). Based on all of the evidences, we were expecting to observe neurological lesions and respiratory symptoms as the classical symptoms of virulent NDV strains. Surprisingly, the virus load at the central nervous system of experimentally infected chickens was low or even zero in qPCR tests, while massive lymphoid depletion and high virus load were observed in studied chickens. In this study, we aimed to identify the molecular basis of pathogenesis of newly emerged NDV-GVII using mRNA profiling of spleen tissues in experimentally infected chickens. To do that, we have focused on cell death related pathways and functional analysis of genes to reveal their potential roles in massive cellular depletion in spleen lymphoid tissues. To our knowledge, this is the first *in vivo* study investigating gene expression profile of this neurotropic velogenic strain.

3.6 Materials and methods

3.6.1 Viruses

The challenge strain used in this study (Mega) has previously been characterised by measuring the MDT index according to standard OIE manual procedures10. The mean death time (MDT) of the isolate was 30h that classified the isolate as virulent or velogenic viruses 11. In brief, ten-fold serial dilution between 10-6 and 10-9 of the virus was made in sterile PBS. A hundred microliters of each dilution were inoculated into the allantoic cavity of each of five 9-day-old embryonated SPF chicken eggs and incubate at 37°C, monitored twice a day for seven days. The time for any embryo deaths was recorded, and the minimum lethal dose that caused death in all embryos was calculated. The minimum lethal dose (MDT) is the highest dilution of the virus that causes death in all the embryos75. In our case, the MDT for the Mega strain of NDV was 30 hours.

3.6.2 Challenge experiment

Animal experiments were performed at the Indonesian research centre for veterinary science (Bbalitvet), Bogor, Indonesia. The animal ethics was approved by the research and animal ethics committee of Bbalitvet institute with the reference number of AH/2015/003. An experienced veterinarian managed the challenge experiment in accordance with the National Health and Medical Research Council (NHMRC) of Australia and the Animal Research Reporting of *In Vivo* Experiments (ARRIVE) guidelines 2.0. Twenty, 1-day old SPF broiler

Ross chickens sourced from Caprifarmindo Laboratories (Bandung, Indonesia) were divided into two groups of 10 and raised in isolator units at biosafety level 3 (BLS3) biocontainment at Bbalitvet. Chickens were allocated randomly into two isolators and tagged individually. At 35 days of age, the birds were inoculated by intraocular and intranasal instillation with100µL of 100 EID50 (Alexander, DJ, Manvell & Parsons 2006; Miller, Patti J et al. 2013) of live Mega strain of NDV (accession number of MN688613) (Doan et al. 2020). One group of 10 birds was inoculated with srtile medium and kept as a negative control in isolator 2.

3.6.3 Tissue collection and RNA extraction

Following the viral challenge, birds were monitored twice a day for clinical signs of ND. Dead birds were collected, and those with severe clinical signs of disease were euthanised and counted as mortalities for that day. Due to death or severe sickness of chickens in the challenged group, the experiment was terminated at 2 day post-infection (2 dpi). The birds in all groups were euthanised by cervical dislocation. After the carcass opening, the spleen was removed, briefly diced and placed in RNAlater (Ambion, Thermo Fisher, MA, USA) for RNA extraction. In total, twenty RNA samples from spleens of challenged and control groups were isolated using a mirVana miRNA isolation kit (Ambion, Thermo Fisher, Lithuania). The isolated RNA was treated with DNase using a DNA-free kit (Thermo Fisher Scientific, Carlsbad, CA, USA). RNA quality was assessed by Agilent 2200 TapeStation instrument, (Agilent Technologies, Santa Clara, CA, USA) and confirmed as RNA Integrity Number (RIN) >5 for all samples.

3.6.4 Detection of virus shedding in challenged and control chickens

From each chicken, a cloacal swab sample was taken, and viral RNA was extracted from cloacal swab samples using QIAamp Viral RNA Mini kit (Qiagen, Louisville, KY, USA) and quantified using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Five microliters of extracted RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Louisville, KY, USA) as per manufacturer's instruction. Absolute quantification for detection of viral load by qPCR was performed using NDV-Fusion Forward: 5′ AAAGTGGTGACACAGGTCGG 3′, and NDV-Fusion Reverse primer: 5′ CCGATGTATTGCCGCTCAAG 3′, generating a 145 bp amplicon. Real-time polymerase chain reaction (RT-PCR) was carried out using the QuantiFast SYBR® Green PCR Kit (Qiagen, Louisville, KY, USA). The reaction was run in an Illumina, Eco Real-Time PCR machine (California U.S.A.) with initial denaturation at 95°C for 3minutes (min) followed by 40 cycles of 95°C for 10 seconds (s) and 60°C for 30 s. Each qPCR reaction was repeated three times in triplicate. The Ct values greater than 35 in viral samples were considered negative (Shirima et al. 2017). One-way analysis of variance (ANOVA) was undertaken to

test for mean differences in *CT* values. The results were analysed in IBM SPSS (v 26.0; SPSS Inc., Chicago, IL).

3.6.5 RNA sequencing

After the initial assessments and the RNA samples' quality control, three RNA samples from the challenged group and three RNA samples from the negative control group with RIN> 5 were selected for the further analysis. The selected samples were submitted to the Australian Genome Research Facility for RNA sequencing. Sequencing libraries were prepared with the TruSeq RNA Library Predation kit as per the manufacturer's protocol and sequenced on Illumina NovaSeq 6000 platform.

3.6.6 Transcriptome analysis

Raw RNA-Seq paired-end reads were checked for quality using FASTQC v0.11.4 (Andrews 2010) and trimmed with TrimGalore v0.4.2 (Krueger 2015) to a minimum length of 150 bp per read and Phred score of 10. Sequencing adapters were removed with AdapterRemoval v2.2.1 (Schubert, Lindgreen & Orlando 2016). Cleaned reads were mapped to the chicken reference genome (GRCg6a) using Hisat2 v2.1.0 (Kim, D, Langmead & Salzberg 2015). Mapped reads were sorted with SAMtools v1.8 (Li, H et al. 2009). Then, sorted mapped reads were summarised using FeatureCounts (Liao, Yang, Smyth & Shi 2014) at the gene level using Ensembl annotation version 97. The Voom-limma pipeline (Liu, R et al. 2015; Ritchie et al. 2015) was used to analyse samples grouped by infection status using the gene-level read counts as input. Briefly, the pipeline involved removing lowly expressed genes, i.e. genes meeting the requirement of count per million (CPM) more than one in at least three samples. The counts were normalised by log-transforming the counts per million to standardise for differences in library size. Counts were also normalised using trimmed mean of M values (TMM) method (Robinson & Oshlack 2010) to avoid bias from different coverage, and samples and individual observational level of each expressed genes were weighted using Voom (Liu, R et al. 2015) to account for heterogeneity in their expression level. Moderated t-statistics tests were used to define differential expression levels between samples. Differentially expressed genes (DEGs) between groups with different infection status were tested and ranked based on the false discovery rate (FDR) less than 0.05.

3.6.7 Pathway analysis of differentially expressed genes

Differentially expressed genes (DEGs) with FDR <0.05 were analysed using the Ingenuity Pathway Analysis software (IPA, QIAGEN, Redwood City, CA, USA), and pathways or functions with z-score >2 were considered to be activated or inhibited (Krämer et al. 2014).

3.6.8 Validation of RNA-Seq data

Applied Biosystems Real-Time PCR System comparative Ct (ΔΔCt) assay was used to validate RNA-Seq results. Gene expression measured in all tissues samples in challenged and control groups (n =20). For each sample, cDNA was prepared from 1 µg of RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Melbourne, Australia) according to the manufacturer's protocols. PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific, Australia) was used to prepare PCR master mix in a 20 µL reaction volume as per the manufacturer's protocol, and 2 µL of the cDNA was added into each reaction well (in triplicate) using a robot (Ep Motion 5075 Robot system, Eppendorf AG, Hamburg, Germany). Thermocycling conditions in ABI Quant StudioTM 6 Flex thermal cycler (Thermo Fisher Scientific, Australia) were polymerase activation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s. A melting curve step from a ramp of 50 to 99 °C was included to assess amplification specificity. Based on their log2 FC (LFC) in RNA-Seq analysis, we selected ten genes that cover the full range of LFC in the comparisons between treatments and control group, and the functional importance of each gene in cell death has also been considered (Galluzzi et al. 2018; Van Herreweghe et al. 2010). These primers are listed in Table 3-1. The primers were designed by NCBI primer tool with amplicons around 100 bp and spanning multiple exons specified also applied to avoid amplification of genomic DNA. uMelt web-based tool used to predict DNA melting curves and the denaturation profile of PCR products for assessing specific amplification of primers (Dwight, Palais & Wittwer 2011). Amplifications of a series of five, ten-fold dilution of cDNA were used to determine PCR amplification efficiencies and correlation coefficients (R) (Hellemans et al. 2007; Laboratories 2006; Pfaffl & Hageleit 2001; Rasmussen 2001). The geometric mean of Ct values of YWHAZ and TBP housekeeping genes that are more stably expressed in the spleen of chicken challenged with pathogens (129, 130) were used to normalise the data. Data were analysed using the Quant Studio Real-Time PCR Analysis software. Replicates of the same sample showing a shifted peak in melting curves were removed. Gene expression was compared between control and treatment groups using the $2(-\Delta\Delta Ct)$ method. Pearson Correlation Coefficient between LFC in qPCR assay and RNA-Seq data was calculated using the GraphPad Prism software version 8.4.2 (GraphPad Software, LLC, San Diego, CA, USA).

Gene Symbol	Primer sequence (5′-3′)	Exon junction (bp)	Fragment size (bp)	Annealing °C	PCR Efficiency (%)	Correlation coefficient (R)	Slop	NCBI accession	Reference
APAF1	F: GGTCAATTGCTGCCAGTTCA	2316/2317 (reverse primer)	94	60	129	0.9539	-2.76	XM_416167.6	this study
	R: TCCTTCAAATCCCAAAGTTTGAT								
CASP3	F: GCAGACAGTGGACCAGATGA	90/91 (reverse primer)	94	60	166	0.9502	-2.349	XM_015276122.2	this study
	R: AGGAGTAGTAGCCTGGAGCA								
CYCS	F: CGTGGGCGCATTTACTGACA	107/108 (forward primer)	81	60	130	0.9650	-2.759	XM_015281453.2	this study
	R: CCGTATGGCACTGGGAACAT								
CASP9	F: CGGAACCTCAAAGCTCAGGAAA	667/668 (forward primer)	99	60	158	0.9524	-2.425	XM_424580.6	this study
	R: ATGGGAGAGGATGACCACGA								
PMAIP1	F: GCCTGCAGAGCGGGAC	114/115 (forward primer)	89	60	130	0.9580	-2.756	NM_001302097.1	this study
	R: GGTTCAGGACTTTCTGCTGC								
TP53INP1	F: ACACTGGCACAACTGGAGG	813/814 (forward primer)	72	60	157	0.9520	-2.429	XM_015282925.2	this study
	R: GGTAGGAAGAGCTGCGACAA								
TP53INP2	F: ATCGAGCTTGGAGAAGAGCC	527/528 (forward primer)	96	60	181	0.9418	-2.227	XM_015296284.2	this study
	R: GGTGACGTAGACGGACATGC								
TP53BP2	F: CTGTGCAAGGAACCTGGTGA	326/327 (reverse primer)	74	60	152	0.9469	-2.483	XM_419394.6	this study
	R: TCGGCTATAGGCCGTTCTGA								
CLTA	F: CTAGCAACAGGGTGGCAGAT	615/616 (reverse primer)	79	60	156	0.9495	-2.440	XM_015280418.2	this study
	R: GCTTCTTCAGCTGCCACATAAC								
MLKL	F: ATTTGAAGGCTGCCCTCTCC	1216/1217 (forward primer)	121	60	206	0.9524	-2.055	XM_015279230.2	this study
	R: GAAGGCCCGACACTGATTGA								
*TBP**	F: CCACGGTGAATCTTGGTTGC	534/535 (reverse primer)	88	60	156	0.9423	-2.447	NM_205103.1	(Khan & Chousalkar 2020)
	R: GCAGCAAAACGCTTGGGATT								
*YWHAZ**	F: TTGCTGCTGGAGATGACAAG	E2/E3 (forward primer)	61	60	120	0.9656	-2.910	NM_001031343.1	(Bagés et al. 2015)
	R: CTTCTTGATACGCCTGTTG								

Table 3-1 Primer sequence used in qPCR for RNA-Seq data validation

For calculating amplification efficiency, a standard curve was generated using a 10-fold dilution of cDNA. The standard curve was created by plotting the Cq values against the log of the template's starting quantity for each dilution. *Used as reference genes for relative expression data analysis. Exon junction represents the spanning of exon on genes sequence.

3.7 Results

3.7.1 Detection of virus shed in the challenged group

To examine effects of the virus on experimentally challenged birds, the Ross broiler chickens were inoculated with a genotype VII NDV. Due to the severe sickness caused by the virus in the challenged group, the experiment was terminated by euthanising all birds at 2 dpi. A cloacal swab has also been taken from all birds, including the control group. The absolute quantification results for detecting the viral fusion gene in samples by qPCR confirmed that all the birds inoculated with NDV-GVII became infected and shed virus at 2 dpi. The mean cycle threshold (*Ct)* value of the challenge group (16.9, SD=1.22) was lower than the control group (41.9 SD=2.92) ($F=967.4$, $df=14$, $p<0.001$), which indicated viral shedding in the challenged group while there was no detection of NDV in the control group.

3.7.2 Gene expression changes induced by NDV infection

Sequencing of constructed libraries from RNA samples resulted in 400 million 100-bp paired-end reads. Similar percentages of reads from each sample (on average 76%) were mapped to the GRCg6a reference genome in the Ensembl database and could be counted as a gene feature by the software Feature Count (Table 3-2).

Sample	Raw count	Cleaned count	Mapping %
Control 1	291,052,64	268,792,54	88.34%
Control 2	113,657,819	102,102,063	87.89%
Control 3	413,589,26	387,238,58	88.54%
Challenged 1	915,321,41	872,570,38	90.42%
Challenged 2	145,682,503	137,041,556	88.67%
Challenged 3	140,667,468	125,491,032	90.23%

Table 3-2 Summary statistics of RNA-Seq output.

The mapping percentage was calculated as the number of reads mapped to the reference genome divided by the number of cleaned count reads.

Of the 24,362 annotated genes in the reference genome, 14,664 (~60%) genes were considered as expressed after our count per million cut-off criteria. By applying the FDR p-value cut-off of 0.05 and log2 fold change more than 1, our analysis revealed 6361 differentially expressed genes (DEGs). 3,506 genes were upregulated, and 2,855 genes were downregulated DEGs (Supplementary Table S1 in Apendix and Figure 3.1). Non-coding RNA transcripts are about 90% of the eukaryotic genome and do not follow the central dogma for genetic information flow in cells. Although several studies aimed to

analyse their existence, a significant challenge exists in their molecular functions and mechanisms of action (Waldron & Lacroute 1975). One of the rapidly expanding fields of this class of transcripts is the long non-coding RNA (lncRNA). A considerable number of transcripts (732) of lncRNA has been detected in our analysis, and interestingly, 513 of these transcripts had the highest (LFC is <-3 or >3) change in expression (Supplementary Table S2 in Apendix). None of the studied genes was included in our analysis due to the lack of a chicken-based biological pathway database for gene expression analysis.

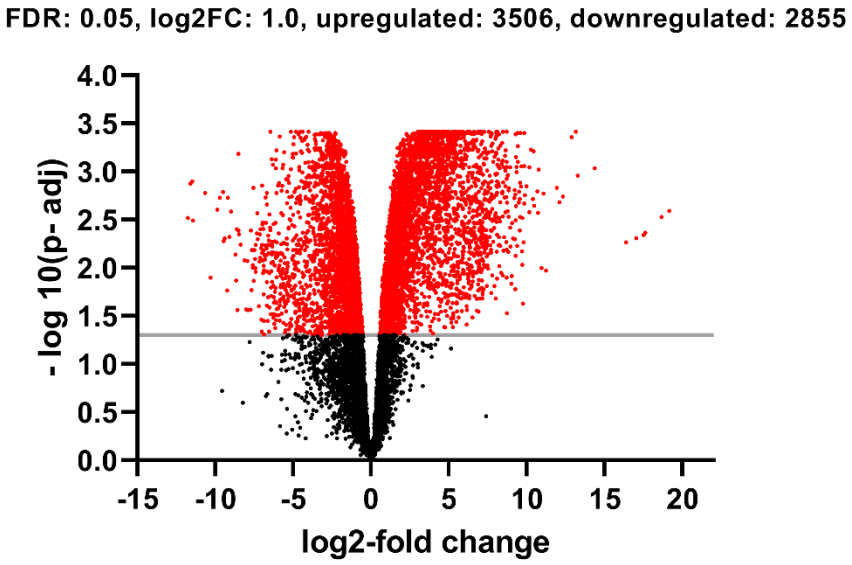

Figure 3.1 The volcano plot of differentially expressed genes between challenged and control birds.

Red dots indicate significantly up-regulated ($p < 0.05$, log2 fold change ≥ 1) and down-regulated genes ($p < 0.05$, log2 fold change ≤ -1). Black dots represent genes.

Based on functional importance genes known to be involved in cell death (Galluzzi et al. 2018; Van Herreweghe et al. 2010), ten genes were selected from our DEGs list to validate the RNA-Seq data. The selected genes were covered the full range of log_2 fold change (log_2 FC) and measured their expression level in qPCRs. The log_2 FC obtained from RNA-Seq data analysis was compared to the log_2 FC obtained in qPCRs. Figure 3.2 shows a comparison between the result of qPCR and RNA-Seq data. The expression patterns obtained from qPCR results for all ten selected genes were similar to their RNA-Seq analysis patterns, with a correlation coefficient (R) of 0.98. These results are confirming the reliability of the RNA-Seq data for gene expression patterns.

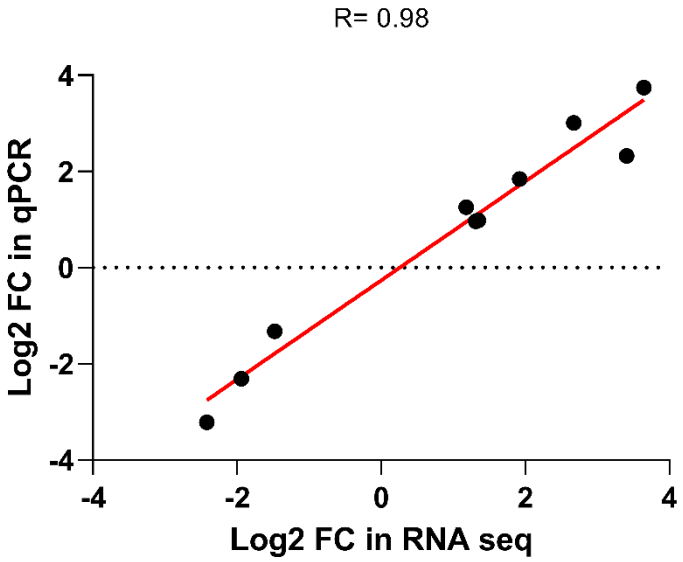

Figure 3.2 Validation of RNA-Seq data using ABI Quant studio qPCR system.

The mean expression of 10 selected genes was calculated by -ΔΔCT method and normalised by mean of Ct values of YWHAZ and TBP YWHAZ as reference genes. The values were converted into log2 fold change (LFC). Each dot point represents one gene. Pearson correlation coefficient test used to compare the results and its value labelled as "R". Plus (+) and minus (-) signs indicate log2 FC values for the upregulated and downregulated genes, respectively.

Functional analysis of 6361 DEGs detected in NDV challenged chickens with IPA indicated the immune response roles (specifically in the early stage of splenic response) to the infection for most of top DEGs with z-score more than 3 (Table 3-3). Due to the use of human- and mouse-based database by IPA for analysis, the types and the functions of some of the chicken genes have not been appropriately indicated. Further investigation into the functions of these uncharacterised proteins and genes would be useful to provide more insight into their contribution to infection.

Symbol	Function of Gene [a]	LFC [b]	FDR [c]	Type(s)	HGNC [d]
AGT	angiotensinogen	14.366	0.0009	growth factor	183
CAMK2A	calcium/calmodulin dependent protein kinase II alpha	10.169	0.0008	kinase	815
CAMKV	CaM kinase like vesicle associated	10.958	0.0101	kinase	79012
ELOVL2	ELOVL fatty acid elongase 2	10.402	0.0019	enzyme	54898
GABRA3	gamma-aminobutyric acid type A receptor subunit alpha3	10.432	0.0006	ion channel	2556
GFAP	glial fibrillary acidic protein	12.885	0.0004	other	2670
GPM6A	glycoprotein M6A	12.11	0.0020	ion channel	2823
IRX1	iroquois homeobox 1	10.723	0.0026	transcription regulator	79192
MMD2	monocyte to macrophage differentiation associated 2	11.067	0.0016	kinase	221938
PACSIN1	protein kinase C and casein kinase substrate in neurons 1	10.774	0.0009	kinase	29993
PADI3	peptidyl arginine deiminase 3	10.267	0.0005	enzyme	51702
PLP1	proteolipid protein 1	13.157	0.0003	other	5354
SLC15A2	solute carrier family 15 member 2	10.478	0.0022	transporter	6565
SLC1A3	solute carrier family 1 member 3	13.277	0.0011	transporter	6507
SLC6A11	solute carrier family 6 member 11	11.937	0.0014	transporter	6538
TTLL2	tubulin tyrosine ligase like 2	10.69	0.0016	other	83887

Table 3-3 The list of the genes that significantly (z-score>3) affected at the challenged group.

a IPA software was used to obtain gene's function from the transcript identifier

b LFC, Log 2 fold change.

c FDR, false discovery rate.

d HGNC, Human Gene Nomenclature Committee

A list of DEGs with consistent responses in expression to NDV infection was released by Zhang *et al*. (2020) (Zhang, J et al. 2020). Comparison of DEGs in our study with Zhang *et al.* revealed 23 shared genes (Table 3-4). Thirteen (56%) of these shared genes had consistent expression change in our study and study of spleen of Hy-Line Brown birds (Zhang, J et al. 2020), spleen (Lan et al. 2010; Rue et al. 2011; Zhang, J et al. 2018), Harderian gland (Deist et al. 2018; Saelao et al. 2018), lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017), Trachea (Deist, Gallardo, et al. 2017a) or embryo (Schilling, Megan A et al. 2018) of Fayoumi or Leghorn chickens challenged with lentogenic NDV. Particularly, interferon induced protein with tetratricopeptide repeats 5 (*IFIT5*) was significantly up-regulated in the spleen of all chickens challenged with virulent and non-virulent NDVs. However, 10 (44%) of these shared significant DEGs had opposite regulation in our study, suggesting an entirely different response to virulent NDV infections.

Gene Name	Function	LFC in this study at 2 dpi	Comparison with Response in other NDV studies
PLCXD1	phosphatidylinositol specific phospholipase C X domain containing 1	0.47	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Harderian gland of Leghorn at 6 dpi (Deist et al. 2018)
SLBP	stem-loop binding protein	0.85	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Spleen (Zhang, J et al. 2018) and trachea (Deist, Gallardo, et al. 2017a) of Leghorn at 2 dpi
OSTM1	osteoclastogenesis associated transmembrane protein 1	1.00	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Leghorn at 2 dpi (Deist, Gallardo, et al. 2017a)
DRAM1	DNA damage regulated autophagy modulator 1	1.40	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Fayoumi and Leghorn at 2 dpi (Deist, Gallardo, et al. 2017a)
PARP12	poly(ADP-ribose) polymerase family member 12	1.48	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Harderian gland at 2 and 6 dpi (Deist et al. 2018), in spleen at 2 dpi (Zhang, J et al. 2018) in Leghorn
SNX10	sorting nexin 10	1.90	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Spleen (Zhang, J et al. 2018) and trachea (Deist, Gallardo, et al. 2017a) of Leghorn at 2 dpi
IFIT5	interferon induced protein with tetratricopeptide repeats 5	6.09	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Spleen of Leghorn at 1 (Rue et al. 2011), 2 (Rue et al. 2011; Zhang, J et al. 2018)and 6 dpi (Zhang, J et al. 2018), and of Fayoumi at 2 dpi (Zhang, J et al. 2018)
P2RX1	purinergic receptor P2X 1	-5.62	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Lung of Fayoumi at 10 dpi (Deist, Gallardo, Bunn, Dekkers, et al. 2017)
KAZALD1	Kazal type serine peptidase inhibitor domain 1	-3.46	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Leghorn at 6 dpi (Deist, Gallardo, et al. 2017a)
HPSE2	heparanase 2 (inactive)	-2.61	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Leghorn at 2 dpi (Deist, Gallardo, et al. 2017a)
UROC1	urocanate hydratase 1	-2.52	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Harderian gland of Leghorn at 6 dpi (Deist et al. 2018)
ROR1	receptor tyrosine kinase like orphan receptor 1	-1.66	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Lung of Fayoumi at 2 dpi (Deist, Gallardo, Bunn, Dekkers, et al. 2017)

FSHR	follicle stimulating hormone receptor	-0.46	Consistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Leghorn at 6 dpi (Deist, Gallardo, et al. 2017a)
AICDA	activation induced cytidine deaminase	-8.98	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Leghorn at 6 dpi (Deist, Gallardo, et al. 2017a)
P2RY8	P2Y receptor family member 8	-3.24	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Harderian gland at 6 dpi (Deist et al. 2018) and trachea at 2 and 6 dpi (Deist, Gallardo, et al. 2017a) in Leghorn
ARHGAP 15	Rho GTPase activating protein 15	-1.85	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Fayoumi at 2 dpi and Leghorn at 2 and 6 dpi (Deist, Gallardo, et al. 2017a)
ASNS	asparagine synthetase (glutamine-hydrolyzing)	-0.58	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Harderian gland of Leghorn at 6 dpi (Deist et al. 2018)
TRIM24	tripartite motif containing 24	-0.57	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Leghorn at 6 dpi (Deist, Gallardo, et al. 2017a)
CDC42SE 2	CDC42 small effector 2	-0.37	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Fayoumi at 2 dpi (Deist, Gallardo, et al. 2017a)
BFAR	bifunctional apoptosis regulator	-0.36	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Fayoumi and Leghorn at 2 dpi (Deist, Gallardo, et al. 2017a)
ST3GAL4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	0.28	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Harderian gland of Leghorn at 6 dpi under heat stress (Saelao et al. 2018)
MYH10	myosin heavy chain 10	0.29	Inconsistent with Spleen of Hy-Line Brown at 2 dpi (Zhang, J et al. 2020) and Trachea of Leghorn at 6 dpi (Deist, Gallardo, et al. 2017a)
EPHB1	EPH receptor B1	0.32	Inconsistent with Spleen of Hy-Line Brown (Zhang, J et al. 2020) and Trachea of Fayoumi at 2 dpi (Deist, Gallardo, et al. 2017a)

Table 3-4 Comparison of DEGs response to NDV in the present study and other in vivo NDV infection studies.

LFC stands for log2 fold change.

3.7.3 Ingenuity pathway analysis of differentially expressed genes

The DEGs list created from transcriptome analysis ($p < 0.05$, log2 fold change ≥ 1), was used as input for IPA analysis. IPA uses a Fisher's exact test p-value cut of 0.05 and an absolute z-score cut-off of 2 or greater for pathways to consider them significantly enriched. In this study, we focused on the pathways engaged in cell death and injury pathways. Overall canonical pathways, upstream regulators, disease and biological functions that were predicted by IPA to be activated are shown in Figure 3.3. Inhibition of IL2 and downregulation of EIF2 signalling as an upstream regulator, resulted in the inhibition of B lymphocytes, the number of cells of the lymphoid system, mononuclear leukocytes and proliferation of cells of the lymphoid system. Inhibition of these processes may deplete immune cells, and lymphatic tissue destruction in spleen observed in the spleen in challenged chicken with virulent NDV-GVII. IPA predicted upregulation of ATF4 that resulted in activation of the synaptogenesis signalling pathway, CREB signalling in neurons and neuropathic pain signalling in dorsal horn neurons in our analysis. Several pathways were significantly impacted by the challenge with virulent NDV as predicted by IPA. Top pathways are shown in Figure 3.4 and a list of all altered pathways provided in supplementary Table S3 in appendix. Overall, many of these pathways lead to cell death and immune response to infection. In particular, Elf2 and mTOR signalling were on top of our downregulated pathways. mTOR signalling activates autophagy and increased autophagy assistances NDV replication (Sun et al. 2014). EIF2 pathway results in viral replication inhibition through the inhibition of translation of viral proteins and increased apoptosis in infected cells (Zhang, S et al. 2014). Reduced autophagy and increased apoptosis would help infected cells with the virus. IPA also predicted activation of signalling by Rho family GTPases, CREB signalling in Neurons and synaptogenesis pathway that are mainly related to this strain's neuropathogenesis.

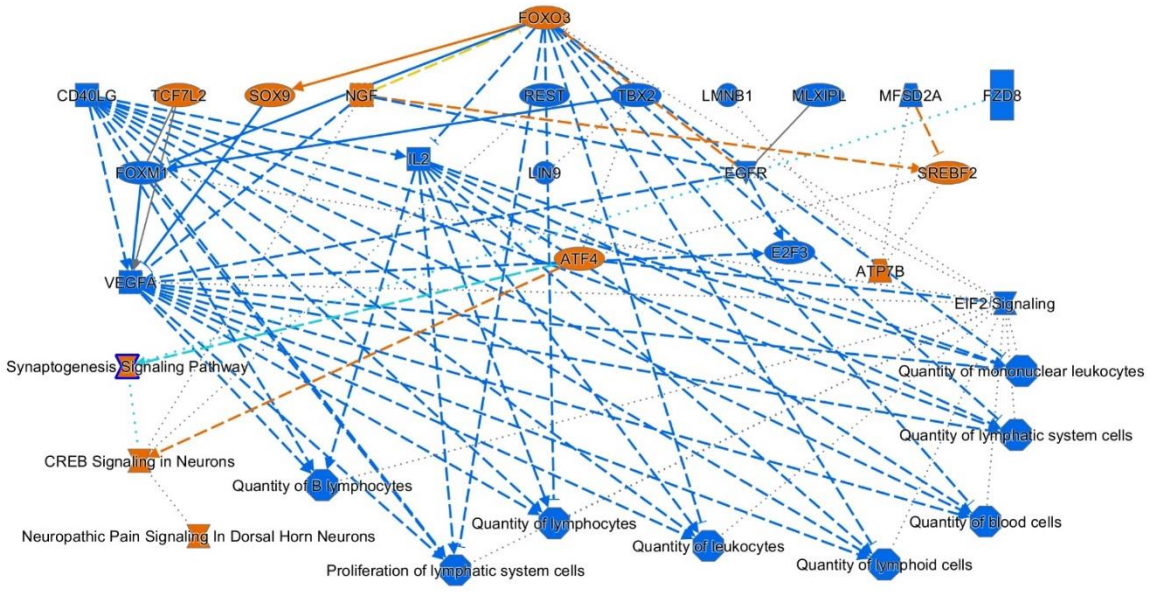

Figure 3.3 Major biological themes

(Pathways, upstream regulators, disease and biological functions) obtained from mapping the significantly upregulated DEGs in the spleen of chicks post-infection. In IPA, only significantly enriched entities that passed a Fisher's exact test p-value cut of 0.05 and passed an absolute z-score cut-off of 2 or greater were visualised. Orange nodes are predicted to be activated (z-score ≥2), while blue nodes are predicted to be inhibited (z-score ≥2). Blue line: leads to inhibition, orange line: leads to activation. Green lines: decreased measurement.

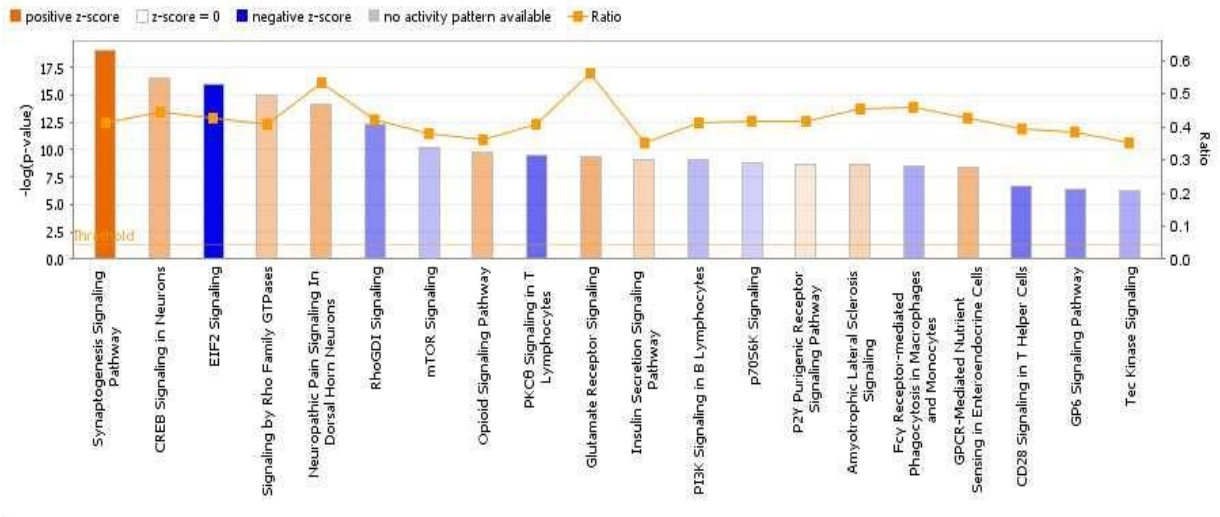

Figure 3.4 Top pathways of differentially expressed genes (FDR < 0.05).

Pathways [Z-score >0.05, -log (p-value) >1.3] in orange predicted to be activated and pathways in blue predicted to be inhibited. The more intensity of the colours, the higher absolute z-score. The orange line indicates the proportion of genes within the pathways that were differentially expressed as ratios. The height of each bar refers to the –log (p-value).

zPathways	z-Score (current study)	IPA prediction in other studies using non-virulent NDV	
		Inhibition	**Activation**
TNFR2 Signalling	-3.00	Spleen (Zhang, J et al. 2020)	Trachea (Deist, Gallardo, et al. 2017a)
TNFR1 Signalling	-2.32	Spleen (Zhang, J et al. 2020)	Trachea (Deist, Gallardo, et al. 2017a)
GP6 Signalling Pathway	-2.23	Spleen (Zhang, J et al. 2020)	Harderian gland (Deist et al. 2018)
Leukocyte Extravasation Signalling	-2.21	Spleen (Zhang, J et al. 2020)	Trachea (Deist, Gallardo, et al. 2017a)
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	-2.02	Spleen (Zhang, J et al. 2020)	Trachea (Deist, Gallardo, et al. 2017a)
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	-1.54	Spleen (Zhang, J et al. 2020)	Trachea (Deist, Gallardo, et al. 2017a)
Tec Kinase Signalling	-1.50	Spleen (Zhang, J et al. 2020)	Lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017), trachea (Deist, Gallardo, et al. 2017a)
B Cell Receptor Signalling	-1.13	Spleen (Zhang, J et al. 2020)	trachea (Deist, Gallardo, et al. 2017a)
Integrin Signalling	-0.92	Spleen (Zhang, J et al. 2020)	Lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017)
IL-8 Signalling	-0.53	Spleen (Zhang, J et al. 2020)	Lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017), trachea (Deist, Gallardo, et al. 2017a)
CD40 Signalling	-0.42	Spleen (Zhang, J et al. 2020)	Trachea (Deist, Gallardo, et al. 2017a)
Thrombin Signalling	-0.12	Spleen (Zhang, J et al. 2020)	Lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017)
IL-6 Signalling	0.18	Spleen (Zhang, J et al. 2020)	Trachea (Deist, Gallardo, et al. 2017a)
P2γ Purigenic Receptor Signalling Pathway	0.88	Spleen (Zhang, J et al. 2020)	Lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017)
Relaxin Signalling	1.00	Spleen (Zhang, J et al. 2020)	Lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017)
Ephrin Receptor Signalling	1.76	Spleen (Zhang, J et al. 2020)	Lung (Deist, Gallardo, Bunn, Dekkers, et al. 2017)

Table 3-5 Comparison of predicted pathways by IPA in the current study with other studies investigating the response to NDV infection. Minus z-Score means inhibition and positive z-Score means activation.

Zhang et al. (2020) study has recently reported 31 pathways with consistent expression response to non-virulent strains of NDV(Zhang, J et al. 2020). Comparison of predicted pathways in our study with impacted pathways by lentogenic NDVs revealed 16 shared pathways (Table 3-5). Significant downregulation (z score < -2) of 5 (31%) of the shared pathways in our study was in agreement with a study of NDV infection caused by a lentogenic strain in spleens of Hy-Line Brown chickens(Zhang, J et al. 2020). However, significant activation of these pathways has been reported in the lung(Deist, Gallardo, Bunn, Dekkers, et al. 2017), Harderian gland (Deist et al. 2018) and trachea (Deist, Gallardo, et al. 2017a) of Fayoumi and Leghorn chicken challenged with a lentogenic strain of NDV. Eleven (68%) of these shared pathways also haven't been significantly impacted (z score > -2 or < 2) by virulent NDV in our study. In contrast, Zhang et al. (2020) and Deist et al. (2017) reported significant activation and inhibition of these pathways, respectively in the response of lentogenic NDV. These findings suggest a different response of the immune system to virulent and non-virulent NDV in different tissues.

Analysis of disease biomarkers in our results revealed functions associated with virulent NDV (Table 3-6). A decrease in proliferation of cells of the lymphoid system, the quantity of B lymphocytes and quantity of mononuclear leukocytes are predicted by IPA using Ingenuity Knowledge Base approach (Krämer et al. 2014). Inhibition of these pathways together may contribute to the massive depletion of lymphoid cells in spleen observed in experimentally infected birds. However, an increase in microtubule dynamics is also predicted to be associated with NDV infection. Upregulation of critical genes such as angiotensinogen (*AGT*) and proteolipid protein 1 (*PLP1*) contributed to activated and inhibited pathways.

Disease and biofunctions	Contributed DEGs for prediction	z-Score	No.
Proliferation of cells of the lymphoid system	***PLP1***, ***SOX2, GAD2, UNC119, APOH, GAD1, MBP, ADCYAP1,*** *FOXJ1,* ***TYR***	-3.571	168
Quantity of B lymphocytes	*SLCO1A2,* ***F3****, ST6GALNAC2,* ***FZD9****, HGF,* ***PLCD1, ESR1****, BST1,* ***ABL1****,* ***STAT1***	-2.769	93
Quantity of lymphocytes tissue	***GAD2****, NPY, SLC4A4, FADS2, FOXC2,* ***PCSK1****, YES1, ESR1* ***ABL1****, MXI1*	-2.402	89
Quantity of mononuclear leukocytes	***AGT, PLP1, GAD2****,* ***MBP****, ADCYAP1, SLCO1A2,* ***F3*** ***SNCA****, NPY,* ***SLC4A4***	-2.785	172
Microtubule dynamics	***AGT, GPM6A, PACSIN1, CAMK2A****, SOX2, PHGDH, RFX4, SLC39A12,* ***GRIN1****, SNCB*	4.311	245

Table 3-6 Top disease and biological function predicted by IPA to be associated with NDV infection.

Bold italic and italic text indicate upregulated and downregulated DEGs, respectively. Genes are sorted ascendingly from left to right based on their fold change. No. means the number of DEGs in our data contributed to disease production.

3.8 Discussion

Understanding the molecular basis of pathogenesis of virulent strains NDVs will provide more reliable information on how these viruses produce unique pathological features in infected chickens, even in vaccinated flocks (Hemmatzadeh 2017). Gene expression pattern analysis helps to understand the virus and host interactions. Several *in vivo* and *in vitro* studies investigated gene expression in different tissues from experimentally infected chickens with different genotypes of NDV (Deist, Gallardo, Bunn, Dekkers, et al. 2017; Deist, Gallardo, et al. 2017a; Deist et al. 2018; Liu, W et al. 2018; Zhang, J et al. 2018; Zhang, J et al. 2020). However, the molecular pathogenesis of genotype VII of NDVs has not been well described mainly through *in vivo* studies. Herein, RNA-Seq and bioinformatics analyses were employed to study spleen transcriptome in experimentally infected birds with highly virulent NDV-GVII.

Gene expression profile analysis of spleen provides insights into host immune defence. Splenic cells produce alpha and beta interferon and interleukin 6 (IL-6) within the first six hours of chickens exposure to virulent NDV (Schroder et al. 2004). Spleen also has a vital role in T cell immune response and lymphocyte transformation in the immune response to NDV infection (Sachan et al. 2015).

In this study, a higher number of differentially expressed genes (>6000) were found when compared to *in vivo* studies of nonvirulent NDV (Deist, Gallardo, Bunn, Dekkers, et al. 2017; Deist, Gallardo, et al. 2017b). Liu et al. (2018) reported 8433 DEGs in chick embryo fibroblasts (CEFs) infected with virulent Herts/33 strain(Liu, W et al. 2018). Regardless of the fundamental differences with our study, both studies showed similar gene expression patterns with a high number of DEGs in response to virulent NDVs.

IPA predicted mTOR and EIF2 signalling inhibition and placed them on top 10 pathways altered by NDV infection in our list. mTOR signalling regulates CD8 T cell differentiation (Araki et al. 2009), and induces Toll-like receptor-mediated IFNA1 in plasmacytoid dendritic cells and has a negative control role in autophagy-mediated cell death after viral infection (Ma et al. 2011; Shrivastava et al. 2012). mTOR signalling activates autophagy and an increased autophagy benefits NDV replication (Sun et al. 2014). EIF2 signalling has been known as a viral replication inhibitor and proinflammatory cytokine expression regulator (Shrestha et al. 2012). EIF2 pathways inhibit translation of virus and increase apoptosis in infected cells, resulting in viral replication inhibition (Zhang, S et al. 2014). Downregulation of these pathways indicates the host's immune response in preventing of viral replication in infected cells. Deist et al. (2017) reported downregulation of EIF2 pathway in lung of challenged Fayoumis with lentogenic NDV at 10 dpi (Deist, Gallardo, Bunn, Dekkers, et al.

2017). However, different activation patterns of the EIF2 signalling pathway were reported in trachea and spleen of challenged birds with non-virulent NDV at 2 dpi (56), and Viral shedding not reported in these studies. Considering the crucial role of these pathways in inhibition of viral replication, downregulation of these pathways along with a considerable virus shedding in our challenged group may indicate altered strategies used by the host to defend itself from the virulent NDVs.

Our IPA analysis also indicated the downregulation of some shared immune pathways with other *in vivo* NDV infections (Zhang, J et al. 2020). IL-8 signalling has a vital role during infectious disease by regulating chemotaxis and activation of neutrophils (Zeilhofer & Schorr 2000). IL-15 production also facilitates homeostasis, development of natural killer cells and CD8 T cells during the anti-viral response (Verbist & Klonowski 2012). Tec kinase signalling pathway has a critical role in response to viral infection and is essential for differentiation and development of CD4+ (146) and CD8+ T cells (Broussard et al. 2006). IL-2 has a critical role in NK cells' activation, lymphocyte proliferation and clearance of intracellular pathogens in chickens (Staeheli et al. 2001; Stepaniak et al. 1999). Inhibition of these share pathways and especially IL-2, as a critical upstream regulator in our study, suggests a suppressed immune response caused by this virulent strains NDV-GVII.

Most of the top upregulated genes indicated in our RNA transcriptome were involved in the immune response to the infection in spleen. AGT and PLP1 both are associated with an increased quantity of cytotoxic CD8+ T-cell (Yu et al. 2005). GPM6A has a role in the expression of human GPM6A mRNA in marginal-zone B lymphocytes expressing human CD27 protein and human IgD complex (Descatoire et al. 2014). Upregulation of AGT and PLP1 in our study contributed to disease production, resulting in massive depletion of the spleen.

Previous studies reported tissue-specific immune response (Deist et al. 2020) and breed-specific immune gene expression in chickens (Schilling, Megan Ann et al. 2019). Our results indicate a small portion and the opposite regulation of shared significant DEGs with previous *in vivo* studies of lentogenic NDV. These differences emphasise virulence as an indicator of the immune response during viral infection.

Our result shows activation of pathways that regulate cellular actin such as signalling by Rho family GTPases resulting in an activated microtubule dynamics pathway. The critical role of this pathway in cell-cell fusion and syncytium formation in the pathogenesis of paramyxoviruses that helps virus entry to the host cell has been reported by Gower et al. (2015) (Gower et al. 2005). These results suggest that NDV, as a paramyxovirus, facilitates viral replication and infection by activating this pathway.

IPA analysis also pointed out the activation of synaptogenesis signalling in our results. This pathway plays a critical role in developing the nervous system by regulating synapse formation between neurons (Martínez et al. 1998). Our results also indicate a decreased quantity of lymphoid tissue and inhibited proliferation of cells of the lymphoid system. This result contrasts with Zhang et al. (2020) that used lentogenic NDV in their challenge experiment (Zhang, J et al. 2020). Activation of pathways that result in the development of synapses in the nervous system and depletion of lymphoid tissue suggests a possible shift in tissue tropism of this strain from a neurotropic velogenic pathogen to a lymphotropic virus. More *in situ* detection and analysis of viral antigens in different tissues of infected birds is necessary to comprehensively understand the tissue tropism of this virulent strains NDV.

On the other hand, our results indicate genes with consistent expression regulation in different studies with many varied experimental factors such as virulence of virus, tissue and breed and time point sampling. This suggests a universal role of these genes in immune response to NDV. One of the most significant genes is IFIT5, an interferon-stimulated gene, that its critical role for innate immune defence against the virus has been confirmed (Zhang, B et al. 2013). IFIT5 recognises and inhibits translation of viral RNA bearing a 5'-triphosphate (Abbas et al. 2013). It also has a key regulator role in activating B-cells by positive regulation of nuclear factor kappa-light-chain-enhancer in NF-κB signalling pathway (Zheng et al. 2015). Overexpression of IFIT5 in transgenic chickens showed significantly enhanced resistance to avian influenza and velogenic NDV (Rohaim et al. 2018). Consistent up-regulation of IFIT5 in the spleen of all chickens challenged with virulent and non-virulent NDV indicates this gene's critical role in the splenic immune response to viral infections.

Compared to the great importance of NDV to the poultry industry and its effects on international trade, there is a relatively modest number of published infectious challenge experiments in which virulent virus has been administered to chickens. *In vivo* experiments using virulent NDV require specialised animal PC3 facilities which are expensive to build and operate. Rapid progression of the induced disease can make it difficult to sample birds over multiple days post-infection. In the present study, we had intended to sample birds at 48 and 72 hours post-inoculation. However, we decided to humanely kill them all at 48 hours post-challenge due to severe clinical signs of disease and high mortality that were already increasing within 24 hours. The reduced quality of RNA extracted from infected birds compared with healthy birds was another limitation of this study that was an unavoidable consequence of the destruction of the host transcriptome during the acute phase of paramyxovirus infection (Suarez et al. 2020).

3.9 Conclusion

This thesis is the first study of gene expression profiling of spleen tissue of experimentally infected chickens with a virulent NDV-GVII. In conclusion, we observed extensive alteration of gene expression in response to this strain in chickens' spleen. Multiple comparisons of the gene expression profile of spleen between this study and previous studies of lentogenic NDV infections indicate differences between DEGs and activation pathway patterns, indicating the role of virus virulence in immune responses. Activation of autophagy-mediated cell death, lymphotropic and synaptogenesis development pathways after viral infection suggest a new tissue tropism for genotype VII NDVs. Further *in vivo* study of these virulent NDV strains in chickens is needed to comprehensively reveal the molecular pathogenesis of these virulent strains virulent strains of NDV.

3.10 Acknowledgment

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3.11 Authors' contributions

MR.: Developed the hypothesis and designed the experimental work, isolated RNA, constructed cDNA libraries, processed and analysed RNA-seq data, wrote the paper. WYL.: supervised the project, reviewed and edited the paper. MMA.: supervised the project, reviewed and edited the paper. YR.: helped with data analysis, reviewed and edited the paper. MIC.: collected samples, reviewed and edited the paper. PTKD.: reviewed and edited paper. ID.: collected samples, reviewed and edited the paper. EDG.: advised on statistical analysis, reviewed and edited the paper. FH.: Acquired funding, supervised the project, reviewed and edited the paper. All authors reviewed and approved the manuscript for publication.

3.12 Additional information

The raw RNA-Seq data have been deposited in NCBI SRA database under the BioProject accession number PRJNA675698

3.13 Competing interests

The authors declare no competing of interests.

3.14 Supplementary information

Supplementary information is provided as Appendix Tables 1-3

4 Necroptosis, necrosis, and oxidative DNA damage in lymphoid tissues of chickens infected with genotype VII Newcastle disease virus

4.1 Statement of authorship

Title of Paper	Repair Assisted Damage Detection and Immunohistochemistry reveal patterns of oxidative stress, necroptosis and tissue tropism in chickens experimentally infected with velogenic Newcastle disease
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Name of Principal Author (Candidate)	Mohammad Rabiei		
Contribution to the Paper	Developed the hypothesis, designed the experimental work, processed and analysed data, wrote and revised the original paper.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	06/02/2021

Name of Co-Author	Milton M. McAllister		
Contribution to the Paper	Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	10/02/2021

Name of Co-Author	Natalie R. Gassman		
Contribution to the Paper	Provided advice on RADD assay. Involved in revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
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Signature		Date	10/02/2021

Name of Co-Author	Farhid Hemmatzadeh		
Contribution to the Paper	Participated in the study design, supervised the project and Involved in the drafting and revising of the manuscript. I give permission for Mohammad Rabiei to present this paper for examination towards the doctor of Philosophy.		
Signature		Date	10/02/2021

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4.2 Abstract

This study was conducted to examine the pathogenesis of virulent genotype VII strains of Newcastle Disease Virus (GVII-NDV), which predominate in Asia and have international importance. Mortality commenced in chicken poults within 1 day of infection, and all birds had to be euthanized by day 2 due to severe clinical illness. The most prominent lesion was massive depletion of all lymphoid tissues. Formalin fixed paraffin-embedded specimens were examined using Immunohistochemistry (IHC) for markers of apoptosis (caspase-3), necroptosis (MLKL), and NDV antigen, and Repair Assisted Damage Detection (RADD) was used to assess DNA damage profiles in the bursa of Fabricius and spleen. Bursal atrophy was associated with profound expression of MLKL, providing evidence that the mechanism of lymphoid depletion involved a non-apoptotic pathway of programmed cell death termed necroptosis. RADD and oxidative RADD analysis of bursa showed a DNA damage pattern consistent with programmed cell death rather than necrosis, in concordance with MLKL expression. In the spleen, MLKL expression was restricted to the central portion of periarteriolar lymphoid sheaths that are closer to the arterioles, while other regions of white pulp expressed neither MLKL nor caspase-3; together with observations of karyorrhexis, fibrinous inflammation, and RADD analyses, we conclude that necrosis was responsible for the majority of lymphoid depletion in the spleen. We speculate that NDV infection may deplete various subsets of lymphocytes by different mechanisms. In addition, the striking occurrence of brown breast muscle in NDV infected birds is suggested to be a result of severe oxidative injury or inhibition of metmyoglobin reductase activity.

4.3 Keywords

Velogenic Newcastle disease virus, pathogenesis, Repair Assisted Damage Detection, Cell death, apoptosis, necroptosis, necrosis, DNA damage, RADD assay.

4.4 Introduction

Newcastle disease virus (NDV) has a worldwide distribution. Because it infects many different avian species, it can be considered a permanent threat to all poultry industries and other aviculture fields (Miller, Patti J et al. 2015). The impact of Newcastle Disease (ND) is very destructive because it causes a very high mortality rate in chickens. Due to ND's economic effect from trade restrictions and limitations, The World Organization for Animal Health (OIE) has ranked ND as a list-A disease with other critical diseases such as *Avian Influenza* (Alexander, D 2000a; Susta et al. 2014). Virulent NDV is a member of the genus avian *orthoavulavirus1* within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* (Dimitrov et al. 2019b; Rima et al. 2019a). This virus was described for the first time in the island of Java (Indonesia) in 1926 and at the same time in Newcastle (England) (Doyle 1927). Various genotypes and strains have been responsible for different ND panzootic outbreaks around the world. Genotype *VII.1.1* (b, d, e, j, l) and *VII.2* (a, h, i, k) caused ND panzootics in Africa, Europe, Middle East and Asia (Dimitrov et al. 2019a; Hemmatzadeh 2017; Xiao, Sa, Paldurai, Anandan, et al. 2012). Phylogenetic analysis of the F and HN genes of NDV isolates indicated that most of the NDVs isolated from Indonesia's clinical cases belong to genotype VII in class II (Berhanu et al. 2010; Dharmayanti et al. 2014; Rabiei et al. 2020; Xiao, Sa, Nayak, Baibaswata, et al. 2012). According to the conventional method based on the clinical signs of NDV infection, strains of NDV have been categorised into five groups (Afonso et al. 2012): (I) Viscerotropic velogenic strains cause acute lethal infections, usually including haemorrhagic lesions in the intestines; (II) Neurotropic velogenic strains causing high mortality with neurological disease followed by respiratory sings without gut lesions; (III) Mesogenic strains causing low mortality with respiratory and neurological signs; (IV) Lentogenic strains causing mild infections of the respiratory tract without any sings in the intestinal tract; and (V) Avirulent strains that replicate in the intestine with no clinical signs; these strains are mainly used as live vaccines (Afonso et al. 2012).

The clinical signs and gross or microscopic lesions are not pathognomonic for ND (Senne 2008). Host related factors such as species, age and immune status as well as virus strain factors such as pathotype, route of infection and dosage can influence the severity of disease and distribution of lesions, and clinical disease might range from subclinical infection to 100% mortality (Alexander, D 1997; Kaleta & Baldauf 1988).

Apoptosis is a hallmark of cytotoxicity in human tumour cell lines infected *in vitro* with NDV, by triggering extrinsic and intrinsic apoptotic pathways (Cuadrado-Castano et al. 2015). *In vitro* and *in vivo* research have shown that NDV can also trigger apoptosis in chicken cells (Kalid et al. 2010; Kommers, G et al. 2002; Ravindra et al. 2008; Robbins & Cotran 2009). Apoptosis occurs both in physiological and pathological conditions and is a multi-pathway process that can lead to programmed cell death. This process relies on the activation of proteolytic caspase enzymes, which denature cytoplasmic proteins and cause the nucleus to fragment. Production of caspase-3, known as effector caspase, is a definitive marker of apoptosis (Robbins & Cotran 2009).

There is another programmed cell death pathway, termed 'necroptosis', that is independent of caspase (Galluzzi et al. 2018). To our knowledge, there have not been any investigations of necroptosis in NDV-infected chickens, but use of NDV in viral oncolytic experiments using human tumour cells has induced cell death by this pathway (Koks et al. 2015; Liao, Ying et al. 2017). The severity of ND is closely correlated with extensive necrosis, especially in infections caused by neurotropic and viscerotropic velogenic strains (Hu et al. 2012).

Viruses are obligate intracellular parasites and use the host cell transcriptome machinery to replicate the viral genome and produce viral protein. This virus-host cell interaction involves DNA transactions, including the induction of DNA damage. Viruses have to deal with the host DNA damage machinery, as host cells limit the DNA damage to constrain and localise the viruses. The host cell uses DNA damage response (DDR) signalling to induce cell cycle arrest to mitigate damage, promote repair, or even induce cell death. Significant DNA damage levels can lead DDR proteins to start apoptotic programmed cell death to preserve host genomic integrity (Ryan, Hollingworth & Grand 2016).

The Innate immune system plays a crucial role in the immune responses to viral infection, and oxidative stress has a crucial impact on the innate immune system. Production of reactive oxygen species (ROS) and prooxidant cytokines during activation of innate immune cells in viral infection enhances the mononuclear phagocytic system (reticuloendothelial system)(Schwarz 1996). Viral infection increases production of oxidants, such as superoxide anion (O_2^-) and nitric oxide (NO). It prevents the synthesis of antioxidant enzymes, such as catalases (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Reshi, Su & Hong 2014). Immune cells need high quantities of antioxidant enzymes compared to other cells, and the limited production and activity of these enzymes lead to a weakened immune response. Production of ROS from granulocytes and macrophages exerts antimicrobial action against many pathogens (Fang 2011). ROS also trigger other pathways to kill or spread viral infections, including autophagy (Huang, J, Lam & Brumell 2011), and apoptosis

(Skulachev 1998). Virus-induced oxidative stress also causes DNA damage by modifying the nucleobases and sugar backbone and results in strand breakages, crosslinking and base loss (Cadet & Davies 2017). If unrepaired, these lesions can be mutagenic and compromise genome integrity. Velogenic and mesogenic strains of NDV have the capability to induce the formation of syncytia. The synthesis of viral HN and F protein at the host cell's surface facilitates syncytia formation (Zeng, Fournier & Schirrmacher 2004).

Most studies have focused on the interface between cellular DDR pathways and infection with DNA viruses (Schmid et al. 2014). However, only a few studies have investigated RNA viruses and DDR (Ryan, Hollingworth & Grand 2016). A recent study investigated DDR in human tumour cells infected with two lentogenic and velogenic strains of NDV (Ren, S et al. 2020). No investigation of DNA damage levels or DDR signalling has occurred in chickens infected *in vivo* with NDV. Our study aimed to investigate highly virulent NDV and evaluate DNA damage associated with oxidative stress and apoptosis and necroptosis patterns in tissues of experimentally infected chickens.

4.5 Material and methods

4.5.1 Challenge experiment

Chicken: The source of chickens was the Caprifarmindo Laboratories (Bandung, Indonesia). Nineteen 1-day old specific-pathogen-free (SPF) broiler Ross chickens were divided into two groups (of 9 or 10) and raised in negative-pressure isolators units at biosafety level 3 (BLS3) biocontainment at the Indonesian Research Center for Veterinary Sciences (Bbalitvet).

Viruses: The genotype VII strains of NDV used in this study (VD and Mega) have previously been characterised as high virulence viruses by OIE criteria (Doan et al. 2020; Rabiei et al. 2020), with mean death-time (MDT) of 33 and 30 hours and accession number of MN699676 and MN688613 respectively. These two strains were both isolated from Indonesia in 2011 and 2013. Virus was propagated in 9-day-old SPF embryonated chicken eggs according to standard OIE manual procedures (OIE 2012). In brief, 0.2 ml of prepared sample was inoculated into the allantoic cavity of three 9-day-old embryonated chicken eggs incubated at 37°C and monitored for 5 days. Embryos died in 24 hours were discarded and, after five days, all eggs were chilled at 4°C for five hours. Allantoic fluid from the dead embryos (after 72 hours) or after 5 days of incubation was tested in Haemagglutination (HA) test. HA positive amnioallantoic fluids were diluted in Brain–heart infusion (BHI) broth and prepared for inoculation.

4.5.2 Pathogenesis experiment

Animal experiments were performed at the Indonesian research centre for veterinary science (Bbalitvet), Bogor, Indonesia. The animal ethics was approved by the research and animal ethics committee of Bbalitvet institute with reference number of AH/2015/003. An experienced veterinarian managed the challenge experiment in accordance with the National Health and Medical Research Council (NHMRC) of Australia and the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0. Chickens were allocated randomly into two isolators and tagged individually. At 35 days of age, 9 birds were inoculated with 10^2 Mean Embryo Infectious Doses (EID_{50}) of either of the previously mentioned strains of live Genotype VII NDV (4 for one and 5 for the other), in 100 μl volume that was divided equally between intraocular and intratracheal sites. Following viral challenge, birds were monitored twice a day for clinical signs of ND. The original plan was to euthanize birds on the third day after inoculation, however due to severe clinical signs and mortality, all remaining birds in the infected group were euthanized by cervical dislocation on the second day. Necropsy examinations were performed on the day of death and tissue specimens for histopathology, immunohistology, and RADD analyses were placed in 10% neutral buffered formalin.

4.5.3 Site detection of NDV in chickens' tissues

From each chicken, tissue samples were taken from spleen, bursa of Fabricius, brain, liver and lung. Viral RNA was extracted from tissue samples using QIAamp Viral RNA Mini kit (Qiagen, Louisville, KY, USA) and quantified using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). 5 μL of extracted RNA was converted to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Louisville, KY, USA) as per the manufacturer's instructions. A conventional PCR method was performed for detection of the NDV-Fusion protein using Forward: 5′ ATGGGCYCCAGACYCTTCTAC 3′, and Reverse: 5′ CTGCCACTGCTAGTTGTGATAATCC 3′ primers, generating a 535 bp amplicon (Liu, H et al. 2011).

4.5.4 Hematoxylin & Eosin staining and Immunohistochemistry

Tissue samples of the spleen, bursa of Fabricius, brain, kidney, lung, cecum, small intestine, pancreas, and proventriculus from negative control and NDV challenged birds were collected in 10% neutral buffered formalin (Sigma-Aldrich, Sydney, NSW, Australia) and within a week of fixation, processed routinely before being embedded in paraffin wax. Thymus was collected when visualised but was not always apparent. Sections (5 μm) were stained by Hematoxylin and eosin (HE) or were subjected to immunohistochemical (IHC) staining for NDV HN antigen, caspase-3 and MLKL antigens using a Dako Omnis Autostainer system.

The Dako machine heat-induced method was applied for antigen retrieval using EnVision FLEX Target Retrieval Solution pH 6.0 for 30 minutes. Antigens were labelled with primary anti-NDV HN monoclonal antibody (Table 4-1) and visualised using DAKO EnVision FLEX/HRP (DAB). Double staining was performed using Anti-Caspase-3 antibody ab4051 or Anti-MLKL antibody MABC604 of tissue samples and visualised by EnVision FLEX Magenta Red. Sections were counterstained with Mayer haematoxylin for 30 seconds, before applying coverslips on DPX mounting media.

Table 4-1 Antibodies used for immunohistochemistry.

Antibody	Clone	pH	Dilution	Pos. Control
Anti Newcastle Disease Virus antibody	8H2, mouse, monoclonal	9.0	1:500	Spleen
Anti- Caspase-3 antibody ab4051	Rabbit polyclonal	6.0 & 9.0	1:250	Tonsil
Anti-MLKL antibody MABC604	3H1 mouse monoclonal	7.4	1:500	Tonsil

4.5.5 Lesion scoring of lymphoid tissues

H&E slides of spleen, bursa of Fabricius, cecal tonsils or other areas of gut-associated lymphoid tissue (GALT) and thymus were examined and analysed by a single veterinary pathologist and scored according to the following scale: 1 = normal dense lymphoid tissue, 3 = normal less dense lymphoid tissue, 5 = equivocal lymphoid lesion suggestive of lymphoid depletion or increased apoptotic bodies, 7 = obvious lymphoid depletion with numerous apoptotic or karyolytic cells, and 9 = massive lymphoid depletion with apoptotic appearance and / or necrotic appearance with karyolysis, often with fibrinous exudate.

4.5.6 DNA assessment using Rapid Assisted Damage Detection (RADD)

RADD and oxRADD assays were used to detect DNA damage within virally infected tissues. FFPE tissues were sectioned and assessed for a broad-spectrum of DNA lesions, i.e., abasic sites, oxidative lesions, pyrimidine dimers, deamination events, and strand breaks, which were labelled using a RADD cocktail (Table 4-2). Additionally, oxidative lesions were specifically examined using a cocktail of FPG, EndoIV, and EndoVIII (oxRADD). Both RADD and oxRADD signals occur predominantly within the nuclei of cells, indicating genomic DNA damage.

Bursa of Fabricius was chosen to investigate RADD for its suitability to analyze types of DNA damage associated with NDV infection. Because histopathological and IHC procedures

were performed before RADD procedures, some of the tissues had been consumed, but enough bursal specimens remained to compare 8 infected with 7 uninfected birds by RADD and then 7 infected with 7 uninfected birds by oxRADD.

Tissues were sectioned in 5 µm thick slices and mounted on poly-lysine coated glass slides. Slides were placed on a heat block set for 65°C and incubated for 8 min to melt the paraffin. Slides were then placed directly in 100% xylene and incubated twice for 10 min each. Slides were rehydrated in water through sequential incubations in ethanol and water mixtures. Specifically, slides were incubated for 5 min each in sequential order of 100% ethanol-0% water; 70% ethanol-30% water; 50% ethanol-50% water; 30% ethanol-70% water; 0% ethanol-100% water. Rehydrated slides were then placed in glass Coplin jars with 200 mL of 10 mM sodium citrate in water and microwaved twice for 2.5 min at 120 watts until the solution reaches 47°C for antigen retrieval. Slides were allowed to cool in water for 2 min. Slides were briefly dried, and tissue samples were outlined with a hydrophobic barrier using a PAP pen. A lesion removal cocktail (Table 4-2) was added to each tissue sample and incubated for 1 h at 37°C. For the full RADD broad-spectrum lesion removal cocktail, all enzymes in Table 4-2 were included. For oxidative lesions only (oxRADD), T4 PDG and UDG were omitted from the lesion removal cocktail and replaced with water. A gap-filling solution (Table 4-2) was then added directly on top of the lesion removal solution and incubated for another hour at 37°C. Slides were washed three times in phosphate-buffered saline (PBS) for 5 min each and blocked in 2% BSA in PBS for 30 min at room temperature (RT). Anti-Digoxigenin (Dig) antibody (Abcam; #ab420 clone 21H8) was incubated at a dilution of 1:250 in 2% BSA in PBS at 4°C overnight. As a negative control for the Dig antibody, an extra slide processed with the full RADD enzyme cocktail was incubated with mouse IgG isotype control antibody (Cell Signaling 5415, clone G3A1) at a dilution of 1:625 at 4°C overnight. This dilution factor matched the µg of anti-Dig antibody used per 100 µL. The next day slides were washed three times in PBS for 5 min each, and Alexa Fluor 546 goat anti-mouse secondary antiserum (Invitrogen) was incubated at a dilution of 1:400 in 2% BSA in PBS for 1 h at R.T. Hoescht 33342 was added at a final dilution of 1:1000 for 15 min at RT to stain the nuclei. Slides were washed three times in PBS for 5 min each, briefly dried, and mounted with coverslips using ProLong Gold Antifade reagent. Slides were allowed to dry overnight in the dark at RT and visualised using a Nikon A1R confocal microscope or stored at 4°C until analysis. The RADD assay was used to detect DNA damage within tissues. A broad-spectrum of DNA lesions, i.e., abasic sites, oxidative lesions, pyrimidine dimers, deamination events, and strand breaks, were detected using the full RADD cocktail. Additionally, a subset of oxidative lesions were specifically examined using an oxRADD cocktail of FPG, EndoIV, and EndoVIII. Both the RADD and oxRADD

signals occur predominantly within the nuclei of cells, indicating genomic DNA damage (Lee et al. 2019).

Table 4-2. RADD sequential reaction conditions.

RADD is performed in two sequential reactions without aspirating reagents between reactions. The lesion processing mix (Left) is placed on prepared tissues and placed in a humidified incubator. The gap filling mix (Right) is added directly to the lesion processing mix and incubated for an additional hour. The reagents are then aspirated and the cells are washed and incubated with anti-digoxigenin antibody.

Lesion Processing Mix	**100 µL total reaction volume**	**Gap Filling Mix**	**100 µL total reaction volume**
UDG (NEB M0280)	2.5 U	Klenow exo$^-$ (Thermo Fisher EP0422)	1.0
FPG (NEB M0240)	4 U	Digoxigenin dUTP (Sigma Aldrich 11093088910)	0.1
T4 PDG (NEB M0308)	5 U	Thermo Pol Buffer (NEB B9004)	10 µL
EndoIV (NEB M0304)	5 U		
EndoVIII (NEB M0299)	5 U		
NAD^+ (100x, NEB B9007)	500 µM		
BSA (Sigma Aldrich)	200 µg/mL		
Thermo Pol Buffer (NEB B9004)	10 L		

4.5.7 RADD Image acquisition

Following protocols established by Lee *et al.* (2019), all images for RADD were acquired using a Nikon A1r scanning confocal microscope with a Plan-Apochromat 10x/0.5 objective.(Lee et al. 2019) Image acquisition settings were obtained by imaging the full RADD samples for tissues and identifying gain settings that limited the number of saturated pixels. These imaging conditions were then used for all tissue imaging allowing for direct comparisons and analysis between tissues. For large tissue sections, images were first mapped using the Acquire Large Image acquisition tool in the Nikon Elements software (NIS-Elements AR 4.51.00), acquired using the 10x objective, and stitched post-acquisition. The tool maps the X-Y-Z positions of individual images within the tissue slice, which are then acquired individually at 10x, 1024x1024 resolution for further analysis. The fluorescent intensity for each 1024x1024 segment is recorded after a binary threshold is applied. Each tissue section required between 4 and 10 1024x1024 section images to cover it completely, depending on the area of the sectioned tissue (Figure 4.1).

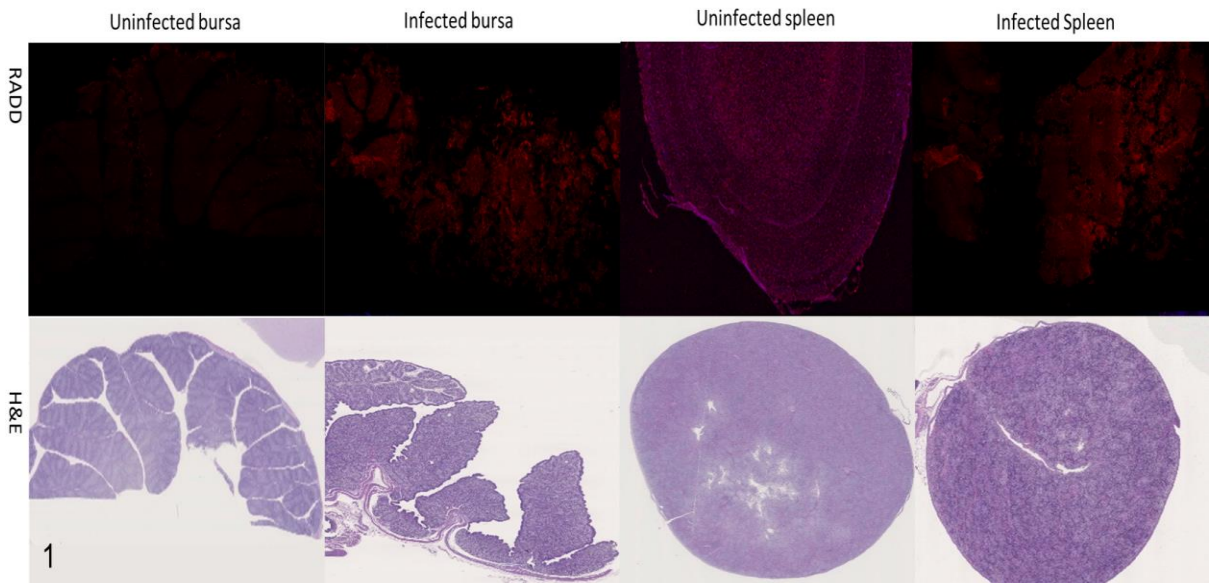

Figure 4.1. RADD assay of DNA adducts within uninfected and infected groups tissues.

RADD (red) and cell proliferation, H&E stain of adjacent tissue slice. Bursa means bursa of Fabricius.

4.5.8 RADD Image analysis

The individual images which make up the large-stitched images, between 9 and 70 images depending on tissue section size, were used for analysis. Each image is 1024 x 1024. The Nikon Elements software was used to create a binary mask of the RADD signal intensity, and the fluorescence intensity was then exported. Gating for the binary mask was defined by the lowest intensity image, and these settings were used for all images. The fluorescence intensity for each individual 1024 x 1024 pixels segment is then averaged over the entire tissue slice to provide the mean fluorescent intensity for the examined tissue section in arbitrary units ± standard error of mean (SEM), and then these values are compared between infected and uninfected groups using Student's t-test.

4.6 Results

In the first instance, tissues from control (N = 10) and challenged (N = 9) birds were histologically processed, embedded in paraffin, and sectioned for histopathology. Additional sections were prepared for immunohistological procedures. Following those procedures, remaining blocks containing bursa were sectioned and examined using full RADD and oxRADD procedures.

4.6.1 Clinical signs and gross lesions

All birds in the challenged group showed severe clinical signs of ND and were severely depressed. Reddened and swollen conjunctiva occurred but this may have been secondary

to ocular instillation of virus due to the local reaction of immune system. Clinical signs included dehydration, ruffled feathers, pale comb, weight loss, anorexia, hunched posture, reduced activity, squinting or closed eyelids, recumbency and death (Figure 4.2). Three of the infected birds were found dead one day after inoculation, and two others were found dead on the second day, when it was decided to euthanize the remaining infected cohort ahead of schedule. All birds in the uninfected control group were euthanized on day 3. All carcasses were necropsied on the day of death in a glovebox using BCL3 procedures; this limited our ability to take quality photographs of gross lesions. Gross lesions were observed only in infected birds. The most pronounced and consistent gross lesions were bursal atrophy and indistinct mottling of spleen. Other gross lesions included thymic atrophy and small numbers of petechial hemorrhages in the mucosa of the proventriculus. An unexpected gross lesion, which occurred in all 9 infected but none of the uninfected birds, was dark discoloration of breast muscle, often with a distinct brown tinge (Figure 4.3).

Figure 4.2 Chicken poults in BSL3 isolators. 1: Healthy birds. 2: Clinically ill birds 1 day post infection with GVII Newcastle Disease Virus

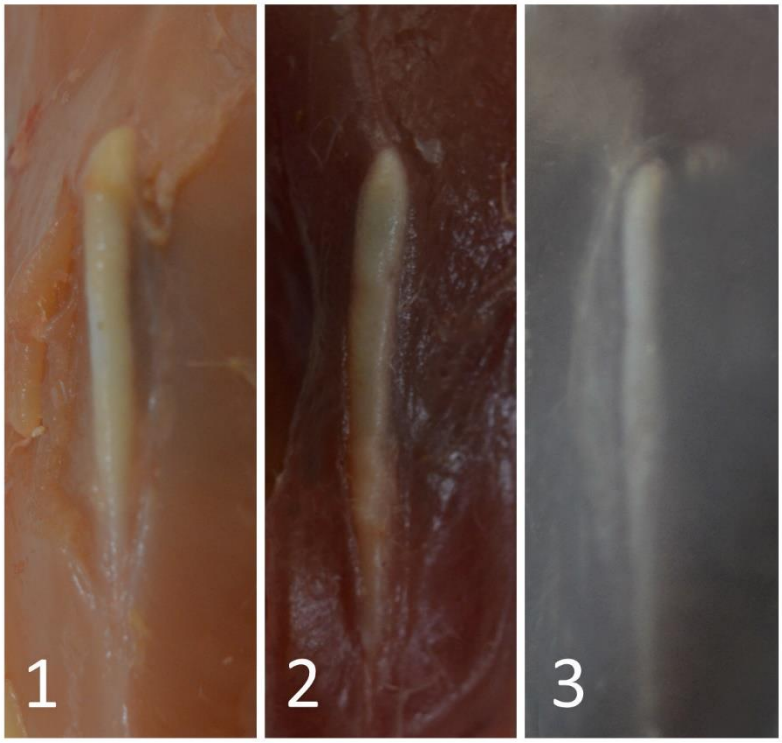

Figure 4.3. 1: Breast muscle of an uninfected bird, 2: Dark breast muscle from an NDV infected bird, 3: Dark brown breast muscle from an NDV infected bird.

4.6.2 Detection of virus in tissues

PCR detection of NDV DNA was positive in 9 of 9 infected birds from spleen, bursa, liver and lung, and in 1 of 9 brains, while PCR results were uniformly negative in all 10 uninfected control birds. Comparable IHC results for viral antigen are reported below. (Figure 4.5 - 4.7).

4.6.3 Histopathology

The 10 birds in the uninfected control group had no significant histopathological lesions. Average lesion scores for total lymphoid tissues of each bird in this group ranged between 1.33 and 2 on a 9 point scale. All 9 infected birds had severe lesions in lymphoid tissues in bursa, spleen, thymus, cecal tonsil and other GALT and Bronchial Associated Lymphoid Tissues (BALT). Spleens had profound lymphoid depletion in white pulp with a combination of nuclear pyknosis and karyorrhexis (Figure 4.4, 4-6, 4-7). Most spleens also had moderate to abundant pink lakes of fibrin that prevented tissue collapse secondary to loss of white pulp. The bursa of Fabricius of infected birds were visibly atrophied and had severe lymphoid depletion with almost complete loss of medullary lymphocytes, moderate to severe loss of cortical lymphocytes, notable reduction in size of follicles and plicae, and relative visual enhancement of the unaffected internal epithelium (Figure 4.4b). In contrast with the spleen, lymphocytes in the bursa only showed pyknotic-like nuclear condensation without

any evidence of karyorrhexis, and there was no fibrinous exudate or other evidence of inflammation. Thymus was often difficult to observe and collect in infected birds due to the subsequent reduction in its substance by age (Kendall 1980) , so only 3 infected thymus were examined. Lymphoid depletion with nuclear karyorrhexis and fibrinous exudate occurred in medullary centres and extended well into lobule cortices, with sparing of about 20% of lymphoid tissue located at the periphery of cortical regions. (Figure 4.4a). Cecal tonsils and other GALT in the proventriculus, small intestine, and colon were also profoundly depleted in all 9 of the NDV infected birds, often associated with vascular congestion or mild mucosal or submucosal haemorrhage. BALT was depleted or necrotic in lungs. Average lesion scores for total lymphoid tissues of the 9 NDV infected birds ranged between 7.5 and 9 on a 9 point scale.

Apart from lymphoid depletion, the most consistently observed lesion was atrphy and depletion and degeneration of fat (Figure 4.8). Lesions in other tissues were variable. Lesions that were observed in some tracheas, lungs, and conjunctiva may have been caused or made worse by intraocular and intra-tracheal routes of viral inoculation. The conjunctiva of the inferior palpebra of some eyes showed hyperaemia or mucosal haemorrhage, up to the inclusion of epithelial erosion, edema, fibrin, heterophilic exudate, and thrombosis. A few tracheas (2) showed oedema in lamina propria, reduction or loss of epithelial cilia, foci of epithelial necrosis, and exocytosis of heterophils. The lung of one infected bird appeared normal, but mild to moderate lesions occurred in other birds including peracute periarterial and intra-airway haemorrhage, periarterial oedema, degeneration and necrosis of BALT, focal attenuation and erosion of bronchiolar epithelium, sloughed cellular debris in airway lumens, scattered degeneration or necrosis of respiratory macrophages, and fibrin thrombi in vascular capillaries of the air capillaries.

Apart from depletion of BALT, gastrointestinal lesions were inconsistent and minor, consisting of congestion or haemorrhage associated with lymphoid depletion. Mild to moderate lesions were observed in the liver of all 9 birds, variably including scattered individual necrotic hepatocytes, small scattered foci of necrosis, patchy vacuolar degeneration of hepatocytes, vascular thrombosis, focal fibrin exudate, and bile stasis. Two birds had small foci of necrosis in exocrine pancreas.

Histopathological lesions were observed in two hearts, including one with a cellular thrombus and one with diffuse myocardial edema. In kidneys, focal tubular epithelial necrosis or autolysis was observed in two birds that were found dead on the second day after infection, and one bird had ectasia of scattered renal tubules which contained droplets and cell debris

in their lumens. Only one bird had brain lesions, consisting of focal venous thrombosis and mild scattered edema.

Skeletal muscles were not included in the experiment's tissue collection protocol, which was created prior to our observation of gross discoloration of breast muscles. Only one specimen of breast muscle was collected and examined (Figure 4.8). Scattered myocytes had condensed hyalinized sarcoplasm or granular disruption and fine vacuolation of sarcoplasm, and small numbers of scattered individual myofibres had nuclear pyknosis.

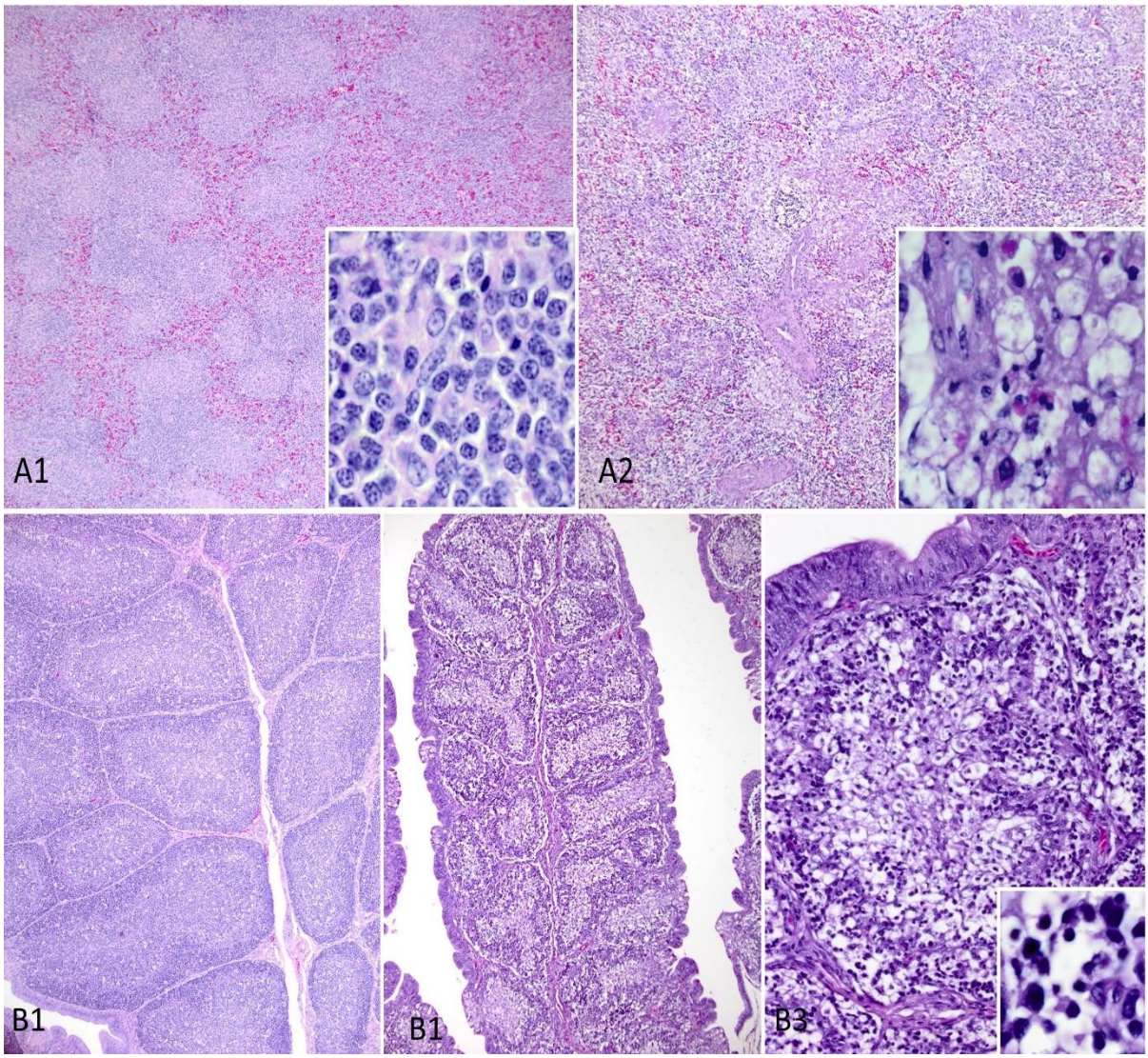

Figure 4.4. Histopathological lesions in spleen and Bursa of Fabricious of control and challenged birds.

A1: Normal spleen from an uninfected chicken poult. Hematoxylin and eosin (HE). Inset: Higher magnification reveals healthy nuclei. A2: Spleen from a chicken poult 2 days after infection with virulent genotype VII Newcastle Disease Virus (GVII-NDV). Lymphoid tissue is severely depleted and contains small scattered lakes of pink fibrin. Inset: Higher magnification reveals dark condensed nuclei and nuclear remnants amidst a proteinacious background exudate. B1: Normal bursa of Fabricius from an uninfected chicken poult. Hematoxylin and eosin (HE). B2: Bursa of Fabricius from a chicken poult 2 days after infection with virulent genotype VII Newcastle Disease Virus (GVII-NDV). HE. B2: Identical magnification as Figure B1, showing profound lymphoid atrophy with reduced size of follicles and plicae, and enhanced visibility of internal epithelium. B3: Higher magnification of the same tissue, showing almost complete absence of medullary lymphocytes and greatly decreased density of cortical lymphocytes in a bursal follicle. Inset: Remnant lymphocytes have condensed pyknotic nuclei without karyorrhexis..

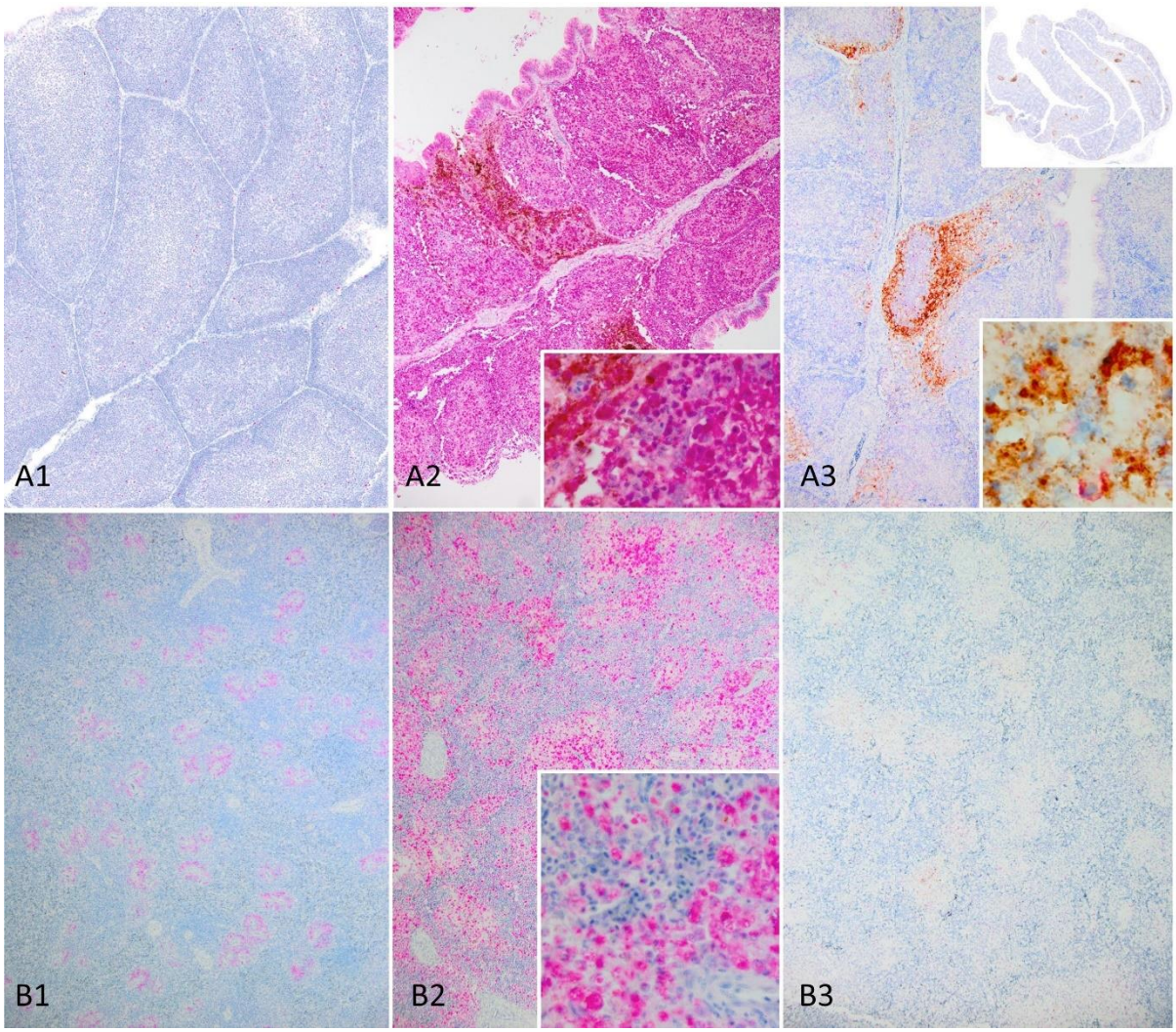

Figure 4.5. IHC result of MLKL and caspase-3 antigens for spleen and Bursa.

A1. Normal bursa of Fabricius from an uninfected chicken poult. Immunohistologic (IHC) stain is negative for expression of MLKL protein. A2: Bursa of Fabricius from a chicken poult 2 days after infection with virulent genotype VII Newcastle Disease Virus (GVII-NDV). Immunohistologic (IHC) stain for MLKL protein (pink) reveals abundant diffuse expression, with scattered follicles staining much darker than others. Inset: Higher magnification showing the staining pattern within the cytoplasm of cells. A3: Dual IHC of the same infected specimen for NDV antigen (brown) and caspase 3 protein (pink, nearly negative). Viral antigen in the bursa is densely expressed in a minority of scattered follicles, with most follicles expressing little or no viral antigen, even though there is a similar degree of profound lymphoid depletion in all follicles. Upper inset: Low magnification shows follicular pattern of viral staining. Lower inset: Higher magnification shows detail of cellular staining. B1: Normal spleen from an uninfected chicken poult. IHC stain for MLKL protein (pink). MLKL is being expressed in a proportion of cells surrounding arteries of the white pulp. B2: IHC stain for MLKL protein in spleen from a chicken poult 2 days after infection with GVII-NDV. In comparison with the uninfected spleen, the amount of cells expressing MLKL antigen is markedly increased. Inset: Higher magnification showing the staining pattern within cells. B3: IHC stain of same infected specimen for caspase 3 protein, showing only minimal expression.

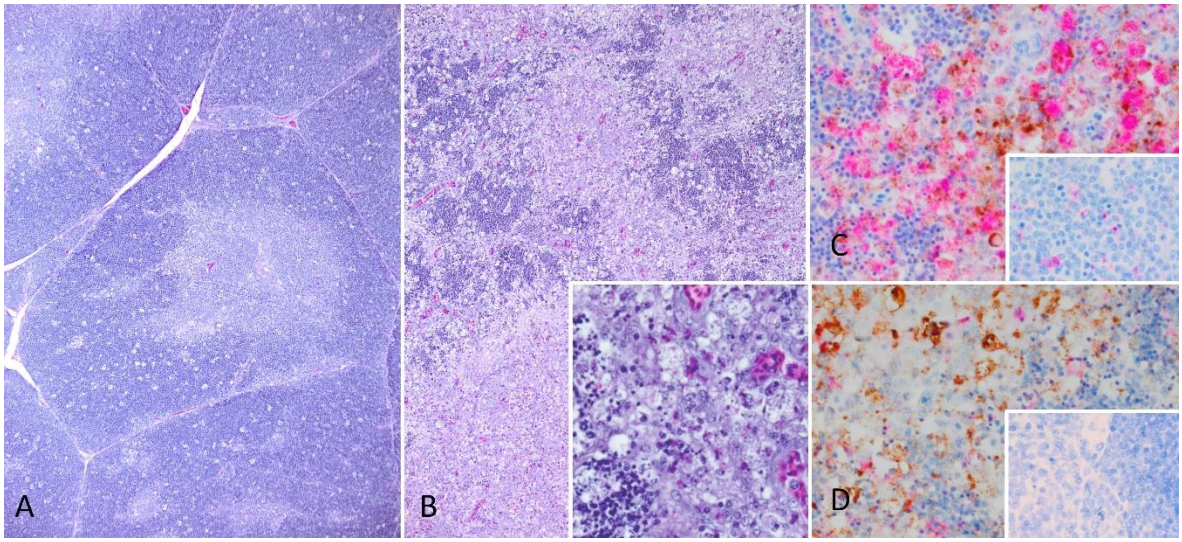

Figure 4.6. Histopathology and IHC of lesions of Thymus in infected and control birds.

A: Normal thymus from an uninfected chicken poult Hematoxylin and eosin (HE). B. Thymus from a poult 2 days after infection with virulent genotype VII Newcastle Disease Virus (GVII-NDV). There is widespread depletion of lymphocytes. HE. Inset: Dark pyknotic nuclei and nuclear remnants of lymphoid cells are visible, with relative sparing of the stellate reticular epithelium. C. Same specimen as in B. Dual immunohistologic (IHC) stain shows NDV antigen (brown) and abundant expression of MLKL protein (pink). Inset: Identical IHC stain of an uninfected control bird, showing absence of viral antigen and a small amount of MLKL expression within scattered lymphoid cells. D. Same specimen as Figs. B and C. Dual IHC stain revealing NDV antigen (brown) and scattered expression of caspase 3 protein (pink). Inset: Identical IHC stain of an uninfected control bird, showing absence of both viral antigen and caspase 3 protein.

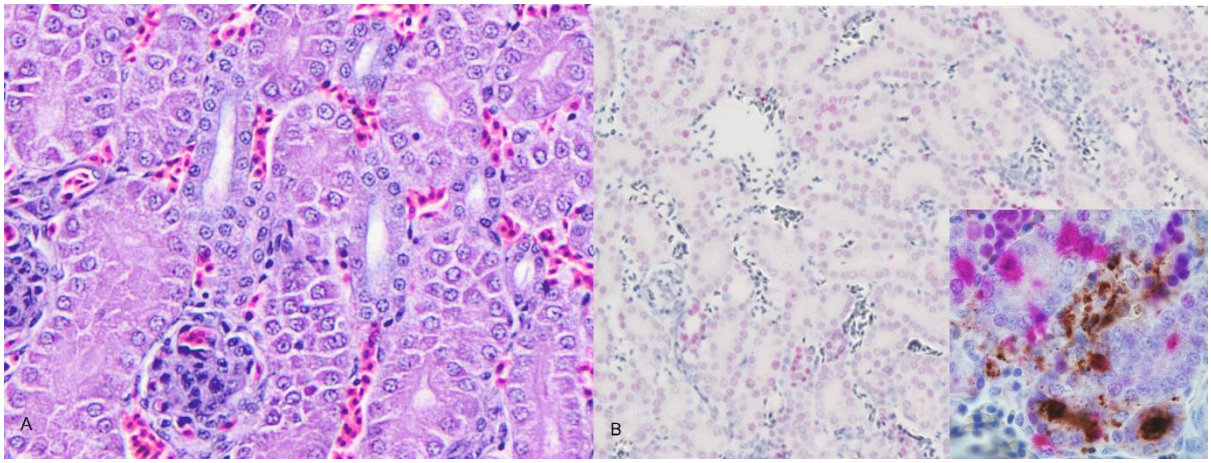

Figure 4.7. Histopathology and IHC of Kidney of infected and control birds.

A: Kidney from a chicken poult 2 days after infection with virulent genotype VII Newcastle Disease Virus (GVII-NDV). Renal tubular epithelium appears morphologically normal. Hematoxylin and eosin (HE). B. A dual immunohistologic stain (IHC) for NDV antigen (brown) and caspase 3 protein (pink) reveals marked expression of caspase in renal tubular epithelium, even though there was no morphological appearance of apoptosis. Inset: Higher magnification shows dark staining of scattered brown viral antigen in blood vessels, renal tubular lumens, and epithelium, indicates viremia and the possibility of renal shedding of virus. In comparison, IHC of kidneys of uninfected control birds (not depicted) did not reveal expression of caspase 3 protein.

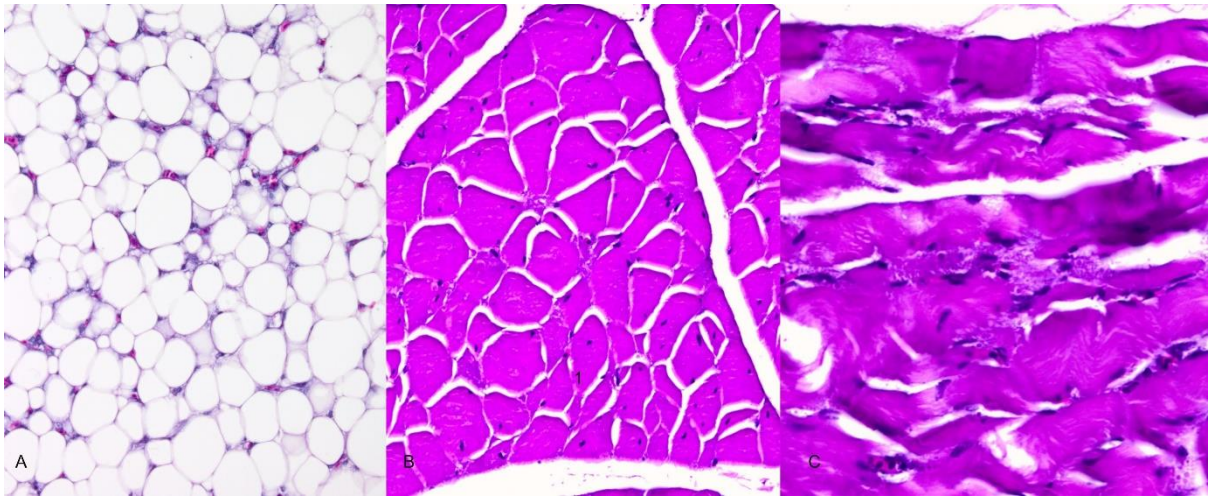

Figure 4.8. Adipose tissue from a chicken poult 2 days after infection with virulent genotype VII Newcastle Disease Virus (GVII-NDV).

A: Showing degenerative changes characterized by uneven reduction in the size of adipocytes and thickening of cell membranes. Hematoxylin and eosin (HE). B and C. Skeletal muscle from a chicken poult 2 days after infection with GVII-NDV. Many myocytes have condensed to hyalinized sarcoplasm, and scattered myocytes have granular degeneration of sarcoplasm, interpreted to be acute diffuse myodeneration. HE.

4.6.4 Immunohistochemical results

Immunohistologic staining for NDV HN antigen was negative in all tissues of all 10 uninfected control birds, but all 9 infected birds had some degree of NDV HN antigen staining in lymphoid and other tissues. The pattern observed in the bursa of Fabricius was dark viral antigen staining of entire follicles that were unevenly distributed among negatively staining follicles (Figure 4.5b), despite the fact that histopathological lesions appeared similar throughout each bursa. The most diffuse and dark staining for viral antigen was observed in the only three thymuses that were collected from infected birds (Figure 4.6). The spleen appeared to have less dense staining for viral antigen than did thymus and bursa. Five kidneys were examined by IHC for NDV HN antigen, and all showed dark staining of scattered foci in blood vessels, renal tubular lumens, and epithelium, suggestive of viremia and the possibility of renal shedding of virus. No evidence of NDV HN antigen was detected in 9 lung, 9 brain, 7 gastrointestinal tract, 7 pancreas, 9 liver or 8 heart of infected birds.

MLKL protein was detected by IHC in tissues of both uninfected and infected birds, however the pattern and amount of staining varied between groups. Uninfected birds had mild background levels of MLKL staining of scattered individual lymphocytes in the bursa of Fabricius (Figure 4.5). In contrast, NDV infected birds had profoundly intense and diffuse

staining for MLKL affecting all bursal follicles (Figure 4.5). MLKL staining of spleens from uninfected birds revealed a light band of staining restricted to the central portion of periarteriolar lymphoid sheaths (PALS) (Figure 4.5), while NDV infected birds had much wider and darker staining with MLKL that often extended and bridged between PALS (Fig. 20a). The thymus of uninfected birds had scattered individual lightly MLKL positive cells, while infected thymus had large dark patches of MLKL positive cells (Figure 4.6). MLKL positive cells in the kidney were rare in both uninfected and infected birds.

Only minor differences, if any, for IHC of caspase-3 protein occurred in lymphoid tissues of uninfected and NDV infected birds, with only modest expression detected in these tissues. Caspase-3 staining of uninfected kidneys showed no expression, but there was nuclear staining of renal tubular epithelial cells in the infected birds, despite the normal appearance of the epithelium and their nuclei (Figure 4.7).

4.6.5 RADD results

RADD procedures were sensitive enough to detect normal background levels of DNA damage in bursal tissues of the uninfected control chickens. Bursas from 7 uninfected chickens had a mean broad spectrum DNA damage level of $5.0 \pm 1.1 \times 10^7$ measured by full RADD with oxidative DNA damage levels at $2.7 \pm 0.73 \times 10^6$ (n=7) as measured by oxRADD. In comparison, bursal tissue from NDV infected birds had significant increases in broad spectrum DNA damage levels ($1.5 \pm 0.35 \times 10^8$, n=8, p = 0.027) and in oxidative DNA damage ($2.6 \pm 0.92 \times 10^7$, n=7, p= 0.044) (Figure 4.9).

As a consequence of performing RADD analyses on specimens of bursa, variable numbers of other tissues that were contained in the same tissue blocks were consequently also subjected to RADD procedures, allowing us to opportunistically examine RADD in other representative tissues, but without statistical power. We examined a small cohort of spleens from uninfected control and NDV infected birds. The full RADD analysis of these spleens showed a higher level of broad spectrum DNA damage within the healthy control birds ($1.6 \pm 0.19 \times 10^8$, n=4) than in the ill NDV infected birds ($6.7 \pm 2.2 \times 10^7$, n=7). The oxRADD showed that oxidative lesions only make up a small portion (< 2%) of the DNA damage observed in the control birds ($2.9 \pm 0.090 \times 10^6$, n=3), indicating that non-oxidative lesions such as deamination events and crosslinks are more prominent in the control tissues. The infected birds showed higher levels of oxidative DNA damage in the oxRADD ($2.1 \pm 0.50 \times 10^7$, n=6) in comparison to the uninfected birds, and when this is compared to the full RADD, oxidative lesions account for approximately 30% of the DNA lesions remaining in the spleens of infected birds.

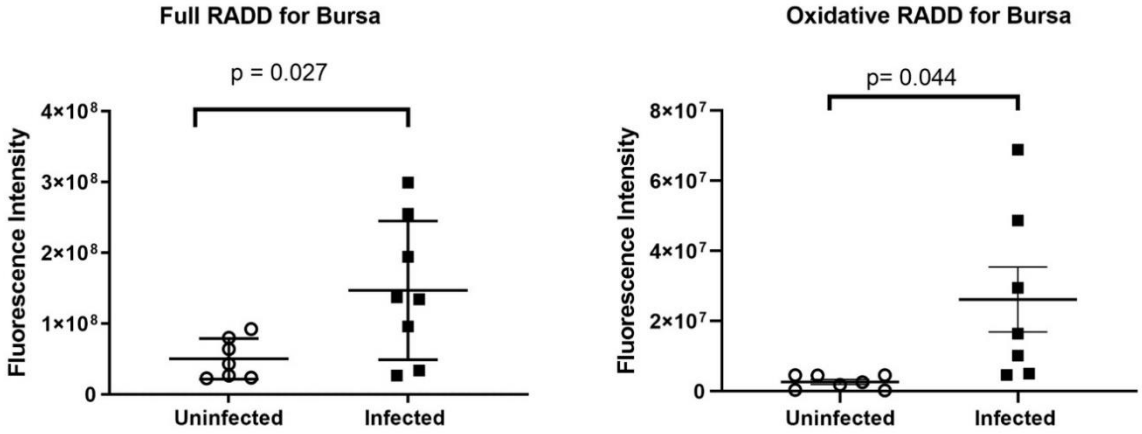

Figure 4.9. RADD and oxRADD result of Bursa of Fabricius in uninfected and infected birds.

Bars represent mean and standard deviation. Bursa means bursa of Fabricius.

4.7 Discussion

Profound lymphoid necrosis was the main lesion induced by infection in our study, and this is in agreement with prior statements about NDV GVII (Hu et al. 2012). We sought evidence to distinguish between different types of programmed cell death (apoptosis or necroptosis) and necrosis in various lymphoid organs. Histopathology of the bursa of Fabricius revealed dark condensed pyknotic lymphocyte nuclei, suggestive of programmed cell death. Immunohistology revealed a very high level of expression of MLKL without caspase-3, which identifies the mechanism of cell death as necroptosis. Histopathological lesions in the spleen of infected birds revealed nuclear changes of lymphocytes ranging between pyknosis and karyorrhexis (nuclear disruption), suggestive of a combination of programmed cell death and necrosis. Spleens also contained fibrinous exudate, indicative of an acute inflammatory process that would be more typical of necrosis than of programmed cell death. Immunohistology of spleens revealed a pattern of increased expression of MLKL in regular narrow bands within PALS, but elsewhere neither MLKL nor caspase-3 had increased expression. These findings in splenic white pulp suggest a larger component of lymphocyte necrosis, admixed with lymphocyte necroptosis in a regional pattern. Lymphoid depletion in the thymus of 3 infected birds was primarily associated with the MLKL marker for necroptosis.

From the above observations, we conclude that lymphoid depletion caused by NDV infection results from a combination of necroptosis (i.e. non-caspase dependent programmed cell

death) and necrosis, with bursal atrophy caused purely by necroptosis. Lymphocyte populations in the spleen are more heterogeneous and include a mixture of mature differentiated cells, in comparison with bursa and thymus which are expected to contain predominant populations of immature or young B or T cells, respectively. It seems likely that different lymphocyte population subsets are more susceptible to either necroptosis or necrosis, but these responses do not appear to divide simply between B and T cells. Further studies are needed to tease out how the different lymphocyte subpopulations are affected by NDV and why these differences occur.

We sought further information about depletion of lymphoid organs using Repair Assisted Damage Detection. Both full RADD ($p < 0.03$) and oxRADD ($p < 0.05$) demonstrated increased DNA lesions within the bursa of Fabricius of infected birds. Viral infection increased the DNA damage load in bursas, and these lesions remained unrepaired, consistent with rapid programmed cell death induced in this tissue, in concordance with the pathological and immunohistochemical findings. The changes observed in both oxRADD and full RADD demonstrate that oxidative lesions increase specifically after viral infection as do non-oxidative lesions such as deamination events, DNA crosslinks and strand breaks. NDV infection has previously been shown to upregulate expression of inducible Nitric Oxide Synthase (Rue et al. 2011), which is an example of a pro-oxidant host response that could contribute to oxidative DNA damage.

RADD and oxRADD patterns in splenic tissue appear to have greater complexity. It is unclear why non-oxidative damage such as uracils and crosslinked DNA lesions were reduced in tissues of infected birds. This finding may suggest viral upregulation of host DNA Damage Response pathways in infected birds, which can benefit replication of RNA viruses (Ryan, Hollingworth & Grand 2016). More work is needed to probe the changes in genomic integrity in spleen and other tissues. Similar to findings in the bursa of Fabricius, oxidative repair pathways of infected birds appear to be compromised in the spleen. This pattern of change is consistent with the possibility of necrosis, tending to support the conclusions of histopathology and IHC.

Other unusual lesions provide evidence of severe metabolic disruption that probably contributed to rapid mortality in the chickens used in this study. Infected birds were thin and had degeneration of fat (Figure 4.8) which is consistent with severe metabolic stress and rapid weight loss. An unexpected observation was the finding of brownish to dark brown breast muscles in the infected birds (Figure 4.3). Muscle can turn brown as a result of oxidation of heme iron in myoglobin to form the brown pigment metmyoglobin, but this occurrence is more commonly observed in stored meats. In living animals the level of

metmyoglobin is normally insignificant, because metmyoglobin is reduced back to myoglobin by metmyoglobin reductase in combination with NADH.(Hagler, Coppes Jr & Herman 1979) The occurrence of brown muscle in living birds is suggestive of the occurrence of severe oxidative injury, either of systemic origin, or perhaps from viral effects upon skeletal myocytes, or possibly secondary to energy depletion and loss of reduced NADH that is necessary for metmyoglobin reductase function. There was no evidence of inflammation in the single histological specimen of breast muscle, although acute myodegeneration was apparent.

Several viruses have been shown to modulate different DNA repair pathways directly, and it appears NDV down-regulates the repair of oxidative DNA lesions specifically. XRCC1, PARP1, and DNA polymerase β are all base excision repair proteins affected by other viral infections. Similarly, nucleotide excision proteins DDB1, XPB, XPC, and XPD are also influenced by viral infections (Hollingworth & Grand 2015). Whether the viral agents act to down-regulate expression, reduce DDR signalling, or alter chromatin structure to limit oxidative lesion removal needs to be determined. Our study demonstrates the persistence of oxidative lesions in the genome of bursa and spleen after viral challenge.

Mesogenic and velogenic NDV infection cause oxidative stress in the brain, liver (Rehman et al. 2018; Subbaiah et al. 2011), and Bursa of Fabricius (Kristeen-Teo et al. 2017b) of chickens. Our results indicate a significant increase in oxidative stress and broad spectrum DNA damage in Bursa of Fabricius induced by NDV infection that is consistent with Kristeen-Teo et *al* (2017) (Kristeen-Teo et al. 2017b).

Infiltration of T cells, especially CD8+ T cells, from spleen to Bursa of Fabricius has been reported following ND (Kristeen-Teo et al. 2017b; Rasoli et al. 2014), Marek's disease (Abdul-Careem et al. 2008), and Infectious Bursal Disease infection (Kim, I-J et al. 2000; Rauf et al. 2011). The different lymphocytic populations in lymphoid organs and infiltration and migration of T cells induced by NDV infection may cause variation in the DNA damage and other DNA damage tolerance profiles across these organs. Infected bursa of Fabricius and thymus accumulate various types of DNA lesions that remain unrepaired at the time of death.

The only organ that showed a substantial increase in apoptosis was kidney, in which tubular epithelium expressed caspase-3, however the histological appearnce of the kidney was normal. These findings may be evidence of systemic shock leading to early renal hypoxia and the onset of caspase expression. There was concurrent uneven distribution of viral antigen within the renal cortex as detected by IHC, so more direct viral mechanisms of apoptosis induction could also be considered.

4.8 Conclusion

This study of NDV infection in chickens convincingly demonstrates that the mechanism responsible for rapid and profound bursal atrophy is necroptosis rather than apoptosis, at least for genotype VII strains, which is a novel observation. Necroptosis also appears to be prominent in the thymus, but depletion of splenic white pulp appears to result primarily from necrosis with a lesser regularly distributed component of necroptosis. We also report the first use of RADD and oxRADD procedures for the study of ND pathogenesis, which reveals increases of oxidative DNA damage in both the bursa and spleen, but with a curious decrease in non-oxidative DNA damage in the spleen. Lastly, we report the unexpected occurrence of dark or brown breast muscle in all NDV infected birds, which is suggestive of profound oxidative injury with excessive *in vivo* formation of metmyoglobin.

4.9 Acknowledgment

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4.10 Authors' contributions

MR.: Developed the hypothesis, and designed and performed the experimental work, and analysis of the data, and wrote the original paper. MMM.: assisted with necropsy, performed histopathology, reviewed and edited the paper. NRG.: helped with RADD assays, reviewed and edited the paper. KJL.: helped with the RADD assay. SA.: helped with RADD assay.. WYL.: reviewed and edited the paper. FH.: Acquired funding, supervised the project, reviewed and edited the paper. All authors reviewed and approved the manuscript for publication.

4.11 Competing interests

The authors declare no competing interests.

5 Discussion

5.1 Introduction

Virulent strains of NDV started from Indonesia in 2012 and now have been reported in the rest of Asia, Africa and South America. It has also been considered a significant biosecurity risk, and sporadic outbreak in ND free countries, including Australia, significantly impacts profitability poultry business. Australia is currently free from virulent strains of NDV. However, the introduction of virulent NDV strains is a severe risk for the Australian poultry industry, one of its major industries. Indonesia is the closest country to Australia and has the second-largest poultry industry in Asia after China (Adi et al. 2010). Wild birds are the natural reservoir of Newcastle disease viruses and ND might occur through spillover from migratory birds from Indonesia (Hubálek 2004). Virulent NDV infection in Australia will almost certainly have substantial economic consequences resulting from high death losses of poultry and other birds, mass culling, and international embargos. Australian poultry industry that worth $6.6 billion per year could collapse, resulting in prolonged poultry shortages (Australia's favourite meat) and egg products will follow by jobless people and social disaster (Wilkinson et al. 2014). This study was funded by the Australian Centre for International Agricultural Research to investigate the molecular pathogenesis of virulent strains of NDV to prepare and support Australia against the possible outbreak.

This thesis has investigated the molecular pathogenesis of virulent genotype VII of NDV in Indonesia. This project's uniqueness is that analysis of this strain's pathogenesis has been investigated through an *in vivo* experiment for the first time. This thesis also has shown that circulating strains in Indonesia are different from vaccine strains, which might be the reason for outbreaks in vaccinated flocks.

5.2 Summary of findings

This thesis examined the molecular pathogenesis of NDV-GVII, divided into two parts: firstly, the full genome sequence of two strains from recent outbreaks have been reported, and secondly, molecular pathogenesis of these strains was investigated in experimentally infected chicken with these strains.

The first research question was "what is the genotype of NDV strains that caused recent outbreaks in Indonesia?" This question has been addressed and investigated in chapter 2 of this thesis.

Tangerang/004WJ/14 and VD/003WJ/11strains have been fully sequenced and reported. Our findings indicated that both strains are similar at the C terminal of the F protein cleavage site. These strains are associated with severe neurological sings in infected chickens with a mean death time (MDT) of 52 and 33 hours, respectively. Phylogenetic analysis of F gene sequences suggests that these circulating strains in Indonesia belong to genotype VII.1 (Figure 2.1). Notably, the amino acid sequence for viral N, P, M, F, HN and L proteins for these two strains have percentage identities of 92%, 81%, 88%, 89%, 85% and 94%, to the *La Sota* strain (GenBank accession no. AF077761.1) respectively. The significant differences in amino acid identities of circulation viruses and *La Sota* vaccine strain suggest the probable reason for vaccine failure and outbreaks in vaccinated flocks. This thesis's findings highlight the urgent need for an update of vaccine development strategies in South-East Asia.

The second research question was “what is the molecular pathogenesis of virulent strains of NDV and which genes and pathways are involved in disease production?”

Chapter 3 addressed this research question using transcriptomic analysis based on RNA-Seq of spleen of chickens challenged with NDV-GVII. Most of the top upregulated genes indicated in our RNA transcriptome were involved in the immune response to the infection in spleen. AGT and PLP1 both are associated with an increased quantity of cytotoxic CD8+ T-cell (Yu et al. 2005). GPM6A has a role in the expression of human GPM6A mRNA in marginal-zone B lymphocytes expressing human CD27 protein and human IgD complex (Descatoire et al. 2014). Upregulation of AGT and PLP1 in our study contributed to disease production, resulting in massive depletion of the spleen. The microscopic findings observed in infected birds in chapter 4 supports gene expression profile analysis and showed profound depletion in spleens of infected birds with NDV-GVII.

This thesis's results also revealed the universal role of *IFIT5* gene in the immune response to NDV. The regulation profile of *IFIT5* in this thesis was consistent with different studies that tested a variety of experimental factors such as virulent of the virus, tissue type and chicken’s breed at different time points after infection. *IFIT5* is an interferon-stimulated gene, and its critical role for innate immune defence against the virus has been confirmed (Zhang, B et al. 2013). IFIT5 recognises and inhibits translation of viral RNA bearing a 5'-triphosphate (Abbas et al. 2013). It also has a key regulator role in activating B-cells by positive regulation of nuclear factor kappa-light-chain-enhancer in NF-κB signalling pathway (Zheng et al. 2015). Overexpression of IFIT5 in transgenic chickens showed significantly enhanced resistance to avian influenza and velogenic NDV (Rohaim et al. 2018). Consistent

up-regulation of IFIT5 in the spleen of all chickens challenged with virulent and non-virulent NDV indicates this gene's critical role in the splenic immune response to viral infections.

IPA analysis of DEGs in this thesis indicated inhibition of CD8 cell differentiation pathways such as mTOR signalling, autophagy and viral replication inhibitor such as EIF2 pathway in challenged chickens. mTOR signalling activates autophagy and an increased autophagy benefits NDV replication (Sun et al. 2014). EIF2 signalling has been known as a viral replication inhibitor and proinflammatory cytokine expression regulator (Shrestha et al. 2012). EIF2 pathways inhibit the translation of viruses and increase apoptosis in infected cells, resulting in viral replication inhibition (Zhang, S et al. 2014). The result of viral antigen detection in spleen tissue compared to Bursa of Fabricious of infected chickens in chapter 4, also indicated non-significant viral distribution in the spleen of infected birds, and supported inhibition of viral replication in spleen.

Our IPA analysis also indicated the downregulation of some shared immune pathways with other *in vivo* NDV infections (Zhang, J et al. 2020). IL-8 signalling has a vital role during infectious disease by regulating chemotaxis and activation of neutrophils (Zeilhofer & Schorr 2000). IL-15 production also facilitates homeostasis, development of natural killer cells and CD8 T cells during the anti-viral response (Verbist & Klonowski 2012). Tec kinase signalling pathway has a critical role in response to viral infection and is essential for differentiation and development of CD4+ (145) and CD8+ T cells (Broussard et al. 2006). IL-2 has a critical role in activating NK cells, lymphocyte proliferation and clearance of intracellular pathogens in chickens (Staeheli et al. 2001; Stepaniak et al. 1999). Inhibition of these share pathways, especially IL-2, as a critical upstream regulator in our study, suggests a suppressed immune response caused by this virulent strains NDV-GVII. The viral antigen detection results in chapter 3 and RNA-Seq result suggest a shift in pathogenesis and tissue tropism of this virulent strains NDV.

RNA-Seq results also show activation of pathways that regulate cellular actin such as signalling by Rho family GTPases resulting in an activated microtubule dynamics pathway. The critical role of this pathway in cell-cell fusion and syncytium formation in the pathogenesis of paramyxoviruses that helps virus entry to the host cell has been reported by Gower et al. (2015) (Gower et al. 2005). Profound lymphoid necrosis was the main lesion induced by infection in our study (chapter 4), and this is in agreement with prior statements about NDV GVII (Hu et al. 2012).

The third research question was" what is the tissue tropism and DNA damage associated with oxidative stress, apoptosis and necroptosis patterns in different tissues of experimentally infected chickens with virulent strains of NDV?"

Velogenic and mesogenic strains of NDV have the capability to induce the formation of syncytia. The synthesis of viral HN and F protein at the host surface facilitates syncytia formation. Syncytia has pro-necrotic potential as leads to a cell-to-cell membrane fusion with the neighbouring cells and cell death (Zeng, Fournier & Schirrmacher 2004).

Histopathological lesions in the spleen of infected birds (chapter 4) revealed nuclear changes of lymphocytes ranging between pyknosis and karyorrhexis (nuclear disruption), suggestive of a combination of programmed cell death and necrosis, and supports activation of syncytia formation in RNA-Seq results.

Recent research highlighted necroptosis as a part of the molecular pathway activated in infected glioblastoma cells with NDV (Koks et al. 2015). Immunohistology of spleens revealed a pattern of increased expression of MLKL in regular narrow bands within PALS, but elsewhere neither MLKL nor caspase-3 had increased expression. These findings suggest a larger component of lymphocyte necrosis, admixed with lymphocyte necroptosis in a regional pattern.

Chapter 3 reported detection of a considerable number of transcripts (732) of lncRNA in our analysis, and interestingly, 513 of these transcripts had high (log_2 Fold Change is <-3 or >3) change in expression (Supplementary Table S2). The induction of lncRNA upon DNA damage has been shown (Sharma et al. 2015). The result of oxidative RADD in chapter 4 reports unrepaired oxidative lesions in the spleen of challenged chicken. This results support each other, indicating the role of LncRNAs in DNA damage. Unfortunately, due to the lack of a chicken-based pathway analysis database, none of these critical lncRNA transcripts were included in our analysis.

Chapter 4 also provided further information about depletion of lymphoid organs using Repair Assisted Damage Detection. Both full RADD ($p < 0.03$) and oxRADD ($p < 0.05$) demonstrated increased DNA lesions within the bursa of Fabricius of infected birds. Viral infection increased the DNA damage load in bursas, and these lesions remained unrepaired, consistent with rapid programmed cell death induced in this tissue, in concordance with the pathological and immunohistochemical findings. The changes observed in both oxRADD and full RADD demonstrate that oxidative lesions increase specifically after viral infection as do non-oxidative lesions such as deamination events, DNA crosslinks and strand breaks. NDV infection has previously been shown to upregulate expression of inducible Nitric Oxide Synthase (Rue et al. 2011), which is an example of a pro-oxidant host response that could contribute to oxidative DNA damage.

RADD and oxRADD patterns in splenic tissue appear to have greater complexity. It is unclear why non-oxidative damage such as uracils and crosslinked DNA lesions were

reduced in tissues of infected birds. More work is needed to probe the changes in genomic integrity in spleen and other tissues. Similar to findings in the bursa of Fabricius, oxidative repair pathways of infected birds appear to be compromised in the spleen. This pattern of change is consistent with the possibility of necrosis, tending to support the conclusions of histopathology and IHC.

Full RADD assay indicated a different pattern of DNA damage in bursa of Fabricius and spleen. The viral infection increased the single and double-strand break in Bursa of Fabricius and remained unrepaired after infection. The differential repair results indicate the viral infection suppresses some DNA repair pathways in bursa while up-regulating in spleen.

The different cell population of chicken immune organs and infiltration and migration of T cells induced by NDV infection (Rasoli et al. 2014), may cause variation in the DNA damage and other DNA damage tolerance profiles across these organs. Infected Bursa of Fabricius and thymus accumulate various types of DNA lesions that remain unrepaired at the time of death. In contrast, the kidney and spleen get only oxidative DNA damage and suggesting that survivable infections could promote mutation and consequently cancer within these tissues long-term.

5.3 Study limitations

Compared to the great importance of NDV to the poultry industry and its effects on international trade, there is a relatively modest number of published infectious challenge experiments in which virulent virus has been administered to chickens. *In vivo* experiments using virulent NDV require specialised animal PC3 facilities which are expensive to build and operate. Rapid progression of the induced disease can make it difficult to sample birds over multiple days post-infection. In the present study, we had intended to sample birds at 48 and 72 hours post-inoculation. However, we decided to humanely kill them all at 48 hours post-challenge due to severe clinical signs of disease and high mortality that were already increasing within 24 hours. The reduced quality of RNA extracted from infected birds compared with healthy birds was another limitation of this study that was an unavoidable consequence of the host transcriptome's destruction during the acute phase of paramyxovirus infection (Suarez et al. 2020).

The well-accepted techniques for measuring DNA strand break and adduct, including comet assays, enzymatic detection, and antibody-based strategies have clear limitations. One significant limitation is that they are often not compatible with archival tissue samples due to the risk of introducing DNA lesions during fixation procedures. In this study, we had only

access to formalin-fixed paraffin-embedded (FFPE) tissue samples. Recently introduced Repair Assisted Damage Detection (RADD) (Lee et al. 2019) system can measure the DNA damage within formalin-fixed paraffin-embedded (FFPE) tissue samples. This study is the first use of the RADD detection system for DNA damage associated with NDV infection.

5.4 Way forward

This thesis has provided an in-depth analysis of the molecular pathogenesis of newly emerged NDV. Chapters 3 and 4 have identified the genes and pathways contributing to disease production. Comparing the transcriptome of time point sampled RNA from different tissues in challenged birds with these strains will shed more light on disease progress and host response. Further studies are also needed to tease out how the different lymphocyte subpopulations in different tissues are affected by NDV and why these differences occur.

We have identified a considerable number of lncRNA transcripts in our analysis. Due to the lack of a chicken-based biological pathway database for gene expression analysis, we couldn’t analyse the role of these high expressed transcripts in our study. Developing a database to analyse chicken biological pathways will help further gene expression analysis.

Further research is needed to investigate direct correlation of neurovirulence genetic marker and pathogenesity and tissue tropism. Viral isolates from different tissues can also be analysed for possible sequence polymorphism. This thesis's findings on the significant differences in amino acid identities of circulation viruses and vaccine strain highlights the urgent need for an update of vaccine development strategies in South-East Asia. The results of this project has a potential implication for better control strategies, improved vaccines and advanced diagnostic tools.

5.5 Conclusion

Full genome sequence of two strains causing the recent outbreak in vaccinated chicken indicated that the circulation viruses has substantially separated from vaccine strains and highlights the urgent need for updated vaccine development strategies in South-East Asia.

This thesis is the first study of gene expression profiling of spleen tissue of experimentally infected chickens with a virulent NDV-GVII. In conclusion, we observed extensive alteration of gene expression in response to this strain in chickens' spleen. Multiple comparisons of the gene expression profile of spleen between this study and previous studies of lentogenic NDV infections indicate differences between DEGs and activation pathway patterns, indicating the

role of virus virulence in immune responses. This study of NDV infection in chickens convincingly demonstrates that the mechanism responsible for rapid and profound bursal atrophy is necroptosis rather than apoptosis, at least for genotype VII strains, which is a novel observation. Necroptosis also appears to be prominent in the thymus, but depletion of splenic white pulp appears to result primarily from necrosis with a lesser regularly distributed component of necroptosis. We also report the first use of RADD and oxRADD procedures for the study of ND pathogenesis, which reveals increases of oxidative DNA damage in both the bursa and spleen, but with a curious decrease in non-oxidative DNA damage in the spleen. Lastly, we report the unexpected occurrence of dark or brown breast muscle in all NDV infected birds, which is suggestive of profound oxidative injury with excessive *in vivo* formation of metmyoglobin.

NDV has been widely used in cancer therapy as an apoptosis inducer oncolytic virus (Tayeb, Zakay-Rones & Panet 2015). This study's finding for potential induction of mutation in tissues also alerts and suggests wisely consideration of NDV in cancer therapy.

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6.1 Appendix 1

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Table S1: All DEG between the challenged and no challenged birds.

Table S2. Differentially expressed long non-coding RNA

Table S3. Significantly impacted pathways by virulent NDV challenge.