Transcriptome profiling of infected chickens with newly emerged genotype VII Newcastle disease virus strain

By

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Abstract

Since it was reported in Indonesia in 1926, Newcastle disease (ND) is an endemic disease in Indonesia resulting in devastating economic losses in poultry. Despite heavy vaccination programs, NDV infection still occurs among commercial chicken farms, including vaccinated birds in Southeast Asia, especially in Indonesia. Therefore, the first objective was to detect and perform full genome sequencing of highly virulent circulating NDVs in vaccinated chicken flocks to identify the most adequate NDV strains as vaccine candidates. Our full genome sequencing analysis on two selected isolates from vaccinated birds in Indonesia has shown that both of them belong to highly virulent NDV-GVII.2 strains, while only 0.4% of the vaccine strains used in this country are genotype VII. Moreover, sequencing analysis of the existing genotype VII vaccine revealed that it has significant differences from GI vaccine strain, which is not sufficiently representative of genotype VII viruses. This finding illustrates that virulent NDV-GVII.2 strains are mainly responsible for the high morbidity and mortality of recent ND outbreaks in poultry in Indonesia.

Virulent NDV-GVII strains were previously characterised as immunopathological phenotypes triggering severe tissue damage probably through apoptosis and necrosis of lymphoid tissues. However, the underlying molecular mechanism of pathogenesis of ND remains to be fully understood. Hence, the second objective was to identify and characterise the molecular mechanism of lymphotropic behaviour of NDV-GVII by focusing on detecting key biomarkers and cellular immune response signalling pathways that contribute to severe lymphocyte destruction in infected birds using RNA sequencing, bioinformatics tools and PPI network analysis. Transcriptomic profiling indicates that virulent NDV-GVII significantly downregulates immunologically regulated genes and innate immune regulating pathways including fMLP signalling in neutrophils, Fcy receptor-mediated phagocytosis in macrophages and monocytes, and leukocyte extravasation signalling and NF-kB activation by

viruses. These transcriptional changes may lead to widespread immunosuppression and enhanced replication of the virus. As a result, the host's immune response may be diminished, delayed, incomplete or display overly strong induction that can cause severe tissue damage. This finding also implies that virulent NDV-GVII strains appear to possess the capability to inhibit the induction of immune responses by targeting lymphocytes and destroying these cells, which may be one indispensable factor of the pathogenesis of NDV-GVII. Moreover, PPI network analysis revealed that the top three significantly enriched gene modules were phagocytosis, immune response-related terms, and glutamate receptor signalling pathway. We identified novel genes EGF, LPAR5, AGT, AGTR1, RAC2, CD4, CD3D, IL7R, NPY, GRM3, and GRAP2 as potential biomarkers. This study provides valuable information to help understand novel immune evading mechanisms of highly pathogenic NDV-GVII from a host-pathogen interaction point of view.

Interactions between the virus and the host determine the success of the viral infection. Hence, the third objective was to identify any particular genes of the virus that may have an important role in virulence and pathogenicity by profiling the transcription of virus in infected birds. This study revealed the transcriptional gradient of these highly pathogenic NDV-GVII genes: NP:P:M:F:HN:L, in which there were a slight attenuations at the NP:P and HN:L gene boundaries. Our result also provides a fully comprehensive qRT-PCR protocol for measuring viral transcript abundance that may be more convenient for laboratories where accessing HTS is not feasible.

Thesis Declaration

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List of publications

As a main author incorporated and a co-author not incorporated in the thesis

1. **Phuong Thi Kim Doan**, Cahyono, MI. Rabiei, M. Pandarangga, P. McAllister, MM. Low, WY. Tearle. R. Dharmayanti, I. Tarigan, S. Indriani, R. Ignjatovic, J. Hemmatzadeh, F. (2020). Genome sequences of Newcastle disease virus strains from two outbreaks in Indonesia. Microbiology Resource Announcements, 9:e00205-20
2. **Phuong Thi Kim Doan**, Yan Ren, Rick Tearle, Putri Pandarangga, Wai Yee Low, Mohammad Rabiei, Farhid Hemmatzadeh [Submitted for publication] Transcriptome analysis of chicken spleens reveals a key to the molecular pathogenesis of virulent Newcastle disease virus genotype VII (NDV-GVII).
3. **Phuong Thi Kim Doan**, Wai Yee Low, Yan Ren, Rick Tearle, Farhid Hemmatzadeh (2022). Newcastle disease virus genotype VII gene expression in experimentally infected birds. Scientific Reports. https://doi.org/10.1038/s41598-022-09257-y
4. Pandarangga, P., Cahyono, M. I., McAllister, M. M., Peaston, A. E., Tearle, R., Low, W. Y., **Doan, P.**, Rabiei, M., Ignjatovic, J., Dharmayanti, N., Indriani, R., Tarigan, S., & Hemmatzadeh, F. (2020). Full-Genome Sequences of Two Newcastle Disease Virus Strains Isolated in West Java, Indonesia. Microbiology Resource Announcements, 9(24), e00221-20
5. Rabiei M, Cahyono MI, **Doan PTK**, Pandaranggaa P, Tarigan S, Indriani R, Dharmayanti I, Ignjatovic J, Low WY, Tearle R, McAllister MM, Alsharifi M, Hemmatzadeh F. (2020). Genome sequences of newly emerged Newcastle disease virus strains isolated from disease outbreaks in Indonesia. Microbiology Resource Announcements, 9:e00204-20.
6. Mohammad Rabiei, Wai Yee Low, Yan Ren, Mohamad Indro Cahyono, **Phuong Thi Kim Doan**, Indi Dharmayanti, Eleonora Dal Grande & Farhid Hemmatzadeh (2021).

Indicators of the molecular pathogenesis of virulent Newcastle Disease Virus in chickens revealed by transcriptomic profiling of spleen. Scientific Report, https://doi.org/10.1038/s41598-021-96929-w.

Conference presentations relevant to the thesis

1. **Phuong Thi Kim Doan**, Putri Pandarangga, Rick Tearle, Milton M. McAllister, Farhid Hemmatzadeh (2021) Novel pathogenesis of newly emerged Newcastle disease virus genotype VII (NDV-GVII). MicroSeq 2021 Conference.
2. P.Pandarangga, M.M.McAllister, A.Peaston, **P.T.K.Doan**, M.I.Cahyono, N.P.I.Dharmayanti, F.Hemmatzadeh (2021). Homologous or heterologous antigen for Newcastle disease virus serology tests in Indonesia: That's the question! The 5th Association of Japan-Indonesia Veterinary Education Symposium.

1 CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The poultry industry plays an indispensable role in many countries. It is one of the primary food providers for humans and is a major contributor of gross domestic product for the global community (Scanes 2007). In many developing countries, poultry helps to eradicate to hunger, reduce poverty and promote gender equality (Dolberg 2007; Guèye, E 2000). The poultry of rural families not only contribute eggs and meat production but also generate income (Guèye, E 2000). Additionally, women's role may gradually change in society when they are educated and trained to be involved in poultry production, where men tend to make the key decisions (Guèye, E 2000).

Furthermore, the role of poultry in scientific discoveries must also be considered, especially in development and immunology. The role of B cells was discovered in experiments with chickens. The experiments carried out in embryonated chicken eggs shed the light on vertebrate limb development (Cooper et al. 1966; Tickle 2004; Weiss & Vogt 2011). On the other hand, chickens are also symbols for religion, culture and entertainment in several nations around the world. For example, chickens are one of the most holy animals used important anniversaries in the worship of ancestors in Vietnamese and Chinese culture. In Africa, poultry have a mystical property, with householders believing that chickens showing neurological symptoms like Newcastle disease can protect the family from bad spirits. These chickens, hence, are maintained and their eggs are consumed as magical foods, allow spread of Newcastle disease (Guèye, EHF 1998).

The burden of poultry diseases challenges poverty alleviation and threatens economically significant losses in intensive poultry production. It is estimated that the monthly income of households may be reduced up to 25% under infectious disease conditions in Madagascar (Rist et al. 2015). In Nigeria, viral diseases such as Avian Influenza and Newcastle disease are important causes of economic loss, while Newcastle disease is the most prevalent disease,

causing up to 58% of the recorded outbreaks that lead to the highest economic vulnerability (Sadiq & Mohammed 2017). In Indonesia, Newcastle disease outbreaks during 2009-2015 in commercial chickens, even in vaccinated flocks, caused huge economic losses in poultry production. Viral genome sequencing has confirmed that these NDV strains belong to genotype VII, a prominent highly virulent NDV isolate that is currently worldwide (Doan et al. 2020; Rabiei et al. 2020; Xiao, S. et al. 2012b). This undoubtedly poses threats to the Australian poultry industry as Indonesia is very close to Australia and wild bird migration is impossible to control. Although many studies have been conducted in vitro, changes in the transcriptome of chickens infected by newly emerged virulent NDV-GVII using next generation sequencing in vivo has not been investigated and it is, therefore necessary to do this, to help shed light on the underlying molecular mechanism of disease. Such findings may contribute to vaccine development and help control or prevent this dangerous NDV strain.

Aims and objectives of the present study

The specific aims of this present study are:

- To phylogenetically and genotypically characterise newly emerged NDV-GVII isolates collected from vaccinated flocks by viral genome sequencing.
- To elucidate the underlying molecular mechanisms of immunopathological tropism of virulent NDV-GVII by profiling the splenic transcriptome of experimentally infected chickens, via RNA sequencing (RNA-seq).
- To investigate viral gene expression and its interaction with the host by measuring differentially expressed genes of virulent NDV-GVII in the infected tissue using RNA-seq and quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR).

1.2 Literature review

1.2.1 History of Newcastle disease

ND is not a new threat to poultry and is one of the most important infectious diseases of domestic birds due to its global distribution and potential to cause devastating losses. Although Newcastle disease (ND) was first documented in Java, Indonesia in 1926 and Newcastle-Upon-Tyne, England in 1927 (Alexander, Dennis J 2009; OIE 2018), there is evidence for disease outbreaks in chickens, fowls and pigeons in Europe before 1926, with symptoms similar to ND (Alexander, Dennis J 2001; Lomniczi et al. 1998; Macpherson 1956). The exact origin of ND remains unknown, possibly due to a lack of specific clinical signs of illness and the difficulty in distinguishing between highly pathogenic avian influenza and ND, as both trigger high mortality and similar respiratory syndromes in avian species (Alexander, Dennis J 2001; Capua 2009). Almost 100 years after the first discovery, ND has been spread throughout the world (Palgen et al. 2015) as shown in Figure 1.1.

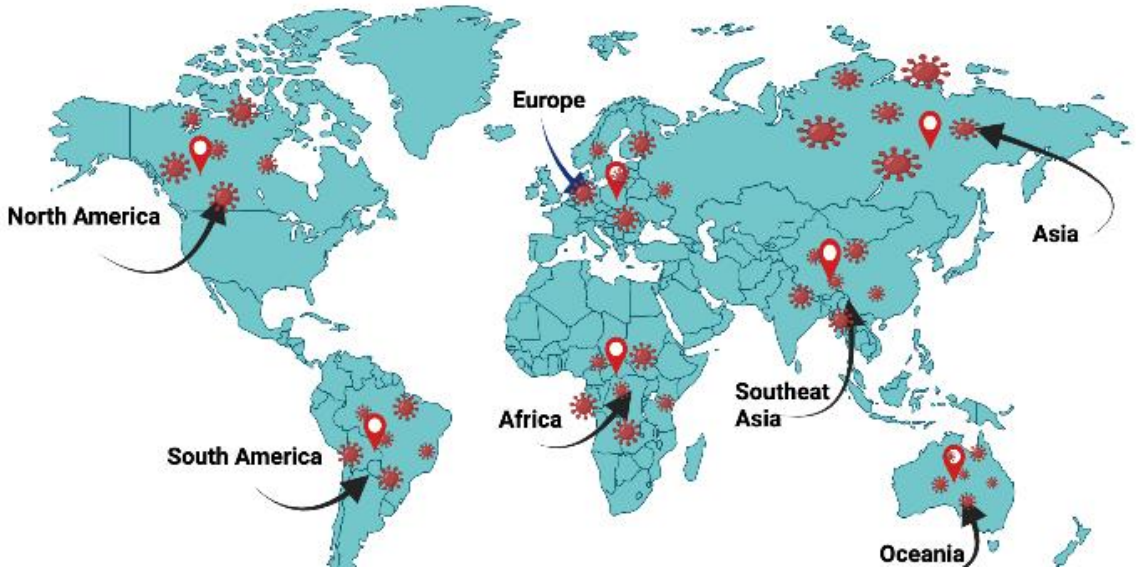


Fig 1.1 The global distribution of Newcastle disease virus

In the last 100 years, the poultry industry has experienced at least five economically devastating panzootic of NDV throughout the world with considerable variation between effected areas in terms of epidemiology and virus strains. The first panzootic was caused by viruses of genotype II, III and IV of class II during the mid-1920s and took approximately 30 years to spread from Southeast Asia to East Asia and Europe (Ballagi-Pordany et al. 1996; Lomniczi et al. 1998). The second panzootic started in the late 1960s in East Asia, and took a significantly shorter time of only 4 years to spread to countries such as the Middle East, Iran, Greece, Europe and the United States (Alexander, Dennis J, Aldous & Fuller 2012; Ballagi-Pordany et al. 1996). This faster speed of spread is likely associated with the increased commercialisation of poultry production globally, along with the wide international trade of captive cage birds, which led to a number of ND outbreaks (Falcon 2004; Pearson & McCann 1975). The numerous studies during 1960s-1970s indicated that the migratory and imported birds were potential reservoirs of velogenic viscerotropic NDV, belonging to genotype V and VI, that was responsible for the second pandemic of ND in different parts of the world, especially in the United States (Lomniczi et al. 1998; Pearson & McCann 1975; Walker, Heron & Mixson 1973). Although some small ND outbreaks caused by other virulent NDV strains were reported somewhere around the world during the 1970s, the true third pandemic of ND, which emerged in the Middle East in the late 1970s and then spread to the Europe during the 1980s, is associated with primary neurotropic manifestations in racing pigeons (Kaleta, E, Baldauf & Alexander 1988; Lumeij & Stam 1985). These pathogenic NDV strains are characterised as pigeons paramyxovirus-1 strains, which was designated as part of NDV sub-genotype VIb, which has greater genetic distance to other NDV strains, being mainly responsible for the third pandemic of ND (Ballagi-Pordany et al. 1996). Subsequently, the poultry flocks in Great Britain were infected by the pigeon virus as a result of consumption of

contaminated food by infected pigeons in 1984 (Alexander, D et al. 1985; Kaleta, E, Baldauf & Alexander 1988).

During the 1980s, Southeast Asia experienced the fourth ND panzootic, triggered by novel virulent genotype VII and VIII ND viruses, and it spread to most countries of Africa, Western Europe and finally South America in 2008 (Cornax, Ingrid et al. 2013; Herczeg et al. 1999; Perozo, Marcano & Afonso 2012). Viruses of genotypes I-IV having a genome size of 15,186 nucleotides emerged in the early 1930s, whereas genotypes V-VIII emerged late 1960s with a genome size of 15,192 nucleotides, the latter consisting solely of virulent viruses (Czegledi et al. 2006). Among the pathogenic ND viruses, NDV-GVII strains are the most frequently isolated from current ND outbreaks in Asia, Middle East, Europe and South America, and are the main agents of a potential fifth ND panzootic (Doan et al. 2020; Lomniczi et al. 1998; Miller et al. 2015; Orynbayev et al. 2018; Perozo, Marcano & Afonso 2012; Suarez et al. 2020; Wajid 2017). The emergence of these novel pathogenic NDV-GVII viruses in poultry farms, especially in vaccinated chickens, has been causing devastating economic losses and negatively impacting poultry industry worldwide.

1.2.2 Newcastle disease virus

Newcastle disease is one of the most severe infectious diseases in poultry. The causative agent, Newcastle disease virus (NDV), is a virus that has capacity to infect over 241 avian species, with the pathogenicity of NDV varying considerably amongst bird species depending on the pathotypes of the virus and the host species (Kaleta, EF & Baldauf 1988). Chickens are the most susceptible to the infection with NDVs, followed by turkeys, ducks and geese, but with the less severe clinical signs and lower mortalities than those reported in chickens (PLAN 2010). Other wild bird species such as pigeons, doves and cormorants are implicated as reservoir species of the highly pathogenic NDV strains but are also very susceptible to the other NDV strains, which is likely to be a transmission risk to the poultry (Alexander, Dennis

J 2009). Specifically, the first outbreak of ND in double-crested cormorants was reported in Canada in 1975, with North America then experiencing another ND epizootic in double-crested cormorants in 1990 (Wobeser et al. 1993). Subsequently, fusion protein sequence analysis from NDV outbreaks in turkeys in North Dakota showed that the cormorant NDV isolates and turkeys NDVs shared the same amino acid sequences, and it is postulated that this wild birds transmitted the viruses to the turkeys (Seal, King & Bennett 1995). The transmission of virus can take primarily place via ingestion and inhalation of virus shed in respiratory secretion or faces by infected birds. It can also be spread via close contact with parts of the carcasses and fomites in the environment. Some virus strains can be transmitted to the hatching chicks through the eggs (Brown, VR & Bevins 2017; Roy & Venugopalan 2005). The clinical presentations of virulent NDV infection depend on the strain (possibly viscerotropic or neurotropic) and the infected host species. However, most of the highly pathogenic strains often result in rapid mortality before gross lesions can be recognized (Brown, VR & Bevins 2017).

NDV is a member of the Avian genus *Orthoavulavirus*, subfamily *Avulavirinae*, family *Paramyxoviridae* (Lefkowitz et al. 2018). It is a single-stranded, non-segmented, negative-sense and enveloped RNA virus that contains six genes which encode six structural proteins, from 3' to 5': nucleocapsid protein (NP), a phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase, named the large polymerase (L) (Kapczynski, Darrell R, Afonso & Miller 2013; Suarez et al. 2020) (Fig 1). The two additional non-structural proteins, V and W(X), are generated by the transcriptional editing of the P gene as shown in figure 1.2 (Qiu et al. 2016; Steward et al. 1993). The NDV genome is approximately 15 kb in length, while the NDV-GVII strains in this study have a genome of 15,192kb, with other NDV strains being 15,186 or 15,198kb in size (Czeglédi et al. 2006; Doan et al. 2020).

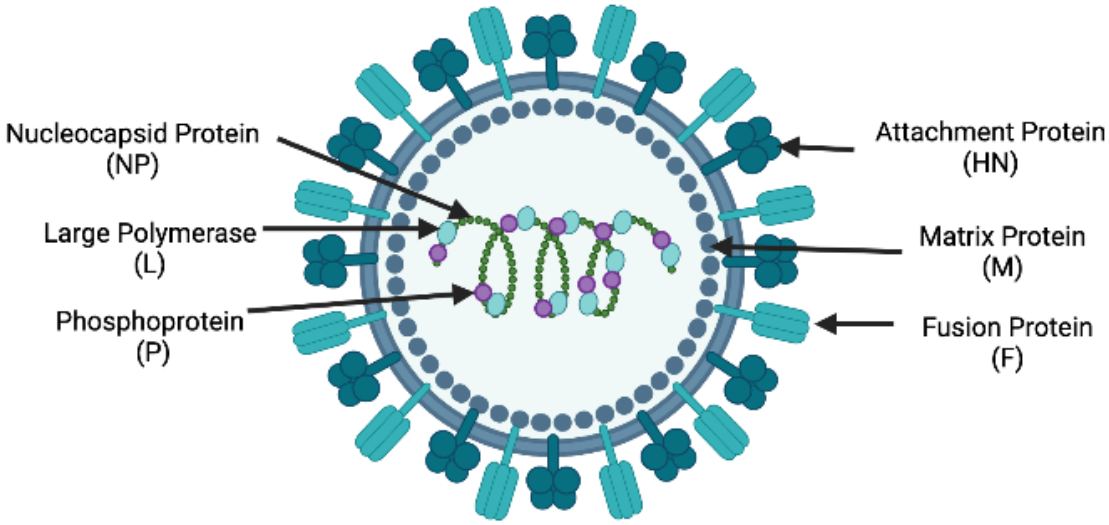


Fig 1.2 Molecular structure of NDV particle

The surface proteins play a crucial role in viral entry into a host cell. Viral infection is initiated by the attachment of viral HN glycoprotein to sialic-acid containing cell surface receptor, leading to fusion of the viral envelope with the host cell membrane through a pH independent mechanism (Chang & Dutch 2012; Suarez et al. 2020). Some studies indicated that while the HN protein may somehow contribute to the virulence of the virus, the F protein, which mediates fusion, is a key determinant of the virulence and viral pathogenicity (Panda et al. 2004; Peeters, BP et al. 1999).

Once viral particles are fused to the host cell, the nucleoprotein complex is able to enter the cell, and the entire transcription process of the negative sense-single RNA genome begins in the cytoplasm of the host cell (Swayne & Glisson 2013). The RNA-dependent RNA polymerase (RdRP) in the viral particles released into the cytoplasm starts producing the positive strand, 5’ capped and 3’ polyadenylated mRNA, from the negative sense RNA genome. Each of viral genes is transcribed from 5’ to 3’ off the coding sequence in a sequential and polar manner (Whelan, Barr & Wertz 2004). As a result of transcription attenuation, higher amounts of mRNA are generated for genes closest to the 5’ region in

comparison with those closer to the 3’ region. The individual genes are then translated into viral protein using host machinery (Suarez et al. 2020). The accumulation of sufficient viral proteins in the host cell allows the negative sense RNA genome to be replicated, which leads to the synthesis of a full length antigenomic RNA, in correlation with the NP protein. Each NP subunit is associated with 6 nucleotides of genomic RNA, hence all NDVs have genome size that is a multiple of six nucleotides (Peeters, B et al. 2000). The antigenomic RNA then serves as a template for the synthesis of full length genomic RNA (Suarez et al. 2020).

Next, the viral nucleocapsid assembly occurs in the cytoplasm of the host cell, involving an attachment mechanism of NP with genomic RNA in order to form the viral helical structure, followed by association with P and L proteins (Dortmans, Jos CFM et al. 2011). The complex is then transported to the plasma membrane where it becomes associated with the F and HN glycoproteins and assembled with M protein as an organizer (El Najjar, Schmitt & Dutch 2014; Takimoto & Portner 2004). Virions are then budded and released from the host cell. The virus is detached from the cell and sialic acid residues are removed from progeny virion by the neuraminidase activity of the HN protein in order to avoid self-aggregation (Dortmans, Jos CFM et al. 2011; Takimoto & Portner 2004). Understanding the viral transcription and replication mechanism is vital to help identify stages that involve the host.

1.2.3 Classification of NDVs and the highly virulent NDV-GVII

All isolates of NDVs are of a single serotype and avian paramyxovirus 1 (APMV-1) isolates can be classified in several ways. In particular, NDV isolates can be divided into distinct lineages and sub-lineages based on the partial nucleotide sequence of the fusion protein (Aldous et al. 2003; Cattoli et al. 2010), or classes and genotypes according based on complete genome sequence (Czeglédi et al. 2006) and F protein cleavage site, respectively (Ballagi-Pordany et al. 1996). NDV strains can also be separated into four pathotypes based on the clinical outcomes produced by infected birds (Alexander, D 2000): (1) avirulent NDV

strains replicate in the intestinal tract with no clinical symptoms, and are mainly used as live vaccines, (2) lentogenic strains are asymptomatic in the intestines and cause mild diseases in the respiratory tract, (3) mesogenic NDVs produce respiratory and neurological disease with low mortality, and (4) velogenic NDV strains causing severe disease with high mortality. Velogenic NDVs can further be divided into velogenic viscerotropic NDVs (vvNDV), which usually cause acute haemorrhagic lesions in the gastrointestinal tract and velogenic neurotropic NDV (vnNDV) strains, which cause marked neurological signs and respiratory infections with no or little gut disease.

Based on the complete F gene sequence phylogenetic analysis, NDVs are divided into two classes (I and II), which shown a large genetic diversity (Dimitrov et al. 2019; Dimitrov, Kiril M, Ramey, Andrew M, et al. 2016). Class I viruses are less diverse with 1 genotype and 3 sub genotypes, whereas class II viruses consist of 21 genotypes (I-XXI) that are the most genetically diverse and cause the most devastating economic losses (Dimitrov et al. 2019). NDV viruses of class II, genotype III-XXI are all virulent, except for genotype X, a low pathogenic strain that have been found from chickens and wild birds (Courtney et al. 2013; Diel et al. 2012; Dimitrov et al. 2019). Among these virulent genotypes, Genotype VII viruses are one of the most genetically divergent and are divided into multiple sub genotypes: VII a, b were virulent trains emerging in the Far East in the 1990s and then spreading to Europe, Asia and South Africa (Aldous et al. 2003). Genotype VII c, d, e represent viruses which were emerged in the 2000s in China, Viet Nam, Kazakhstan, South Africa and Europe, while genotype VII f, g, h were isolated in Africa and Southeast Asia (Aldous et al. 2003; Bogoyavlenskiy 2009; Snoeck et al. 2009; Wang, Z et al. 2006; Xiao, S. et al. 2012b). Recently, genotype VIIi, which has been reported in Israel, Pakistan, Iran and India, is closely related to Indonesian viruses isolated from 1983 (Desingu et al. 2017;

Ghalyanchilangeroudi et al. 2018; Miller et al. 2015). A new sub genotype, genotype VIIj, was isolated in Shandong and Jiangsu provinces, China. Thus NDV-GVII shows high genetic diversity and complexity (Xue et al. 2017).

Nevertheless, the utility of multiple naming systems with different approaches and insufficient objective criteria, along with constant NDV evolution, may cause confusion as well as difficulties in classification. Therefore, much effort has been made to determine the most useful universal classification system (Aldous et al. 2003; Diel et al. 2012). This unified classification system developed, based on the sequence of complete fusion protein of all presented NDVs and nucleotide distance estimation using phylogenetic neighbour-Joining, Maximum-likelihood and Bayesian methods (Dimitrov et al. 2019). The F protein gene was used for comparative analysis as it is one of the key antigenic determinants of APMV-1, which is more likely to show genetic variation than other proteins (Aldous et al. 2003).

In this document, the newly unified phylogenetic classification criteria will be applied because it is up to date and the most commonly used – see Table 1.1 (Aldous et al. 2003; Dimitrov et al. 2019). According to Dimitrov et al (2019), genotype VII is now grouped into three sub genotypes: genotype VII.1.1 including the former sub genotype VII b, d, e, j, l and was responsible for the fourth ND panzootic, whereas the fifth NDV panzootic is associated with genotype VII.2 consisting of VII a, h, i, k in Africa, Europe, Middle East, Asia. Genotype VII.f is an exception and subsetted into a single sub genotype VII.1.2. Using the unified and standard dichotomous naming system makes it easier for scientists to track the ancestry of the viruses and avoid confusion in classification.

Table 1.1. The distribution of Newcastle disease virus genotype VII

Sub-genotypes	New classification	Host	Year	Countries
VIIa	VII.2	poultry	1990s	Europe, Taiwan, Indonesia, Western China
VIIb	VII.1.1	poultry, wild birds	1998-2014	China, Viet Nam, Israel, Europe, Turkey, South Africa, Mozambique, Kazakhstan, the Far East, the Middle East, India
VIIc	-	poultry	1996-1997	Europe
VIId	VII.1.1	Poultry, wild birds	1998-2013	China, South Korea, Colombia, Israel, South Africa, Ukraine and Venezuela, Europe, Kazakhstan, Viet Nam
VIIe	VII.1.1	Chickens, domestic waterfowl	1997-2014	China, Japan, Taiwan, Viet Nam
VIIf	VII.1.2	Domestic poultry, pigeons	1996-2008	China
VIIi	VII.2	Chickens, pheasants, peafowl	2010-2015	Indonesia, Israel, Pakistan, Eastern Europe, Iran
VIIj	VII.1.1	chickens	2009-2015	China
VIIh	VII.2	Chickens, Wild birds	2007-2015	Indonesia, Malaysia, China, Vietnam, Cambodia, South Africa, Mozambique, Botswana, Malawi, Zambia
VIIk	VII.2	chickens	2016	Namibia
VIIl	VII.1.1	chicken	2011	Iran
VIIg	-	chicken	1996-2015	China, Iran

1.2.4 Defining and assessing NDV pathogenicity

Virulence is often used interchangeably with pathogenicity. Virulence refers to characteristics of the pathogen alone, while pathogenicity incorporates interactions between the pathogen and the host. Virulence is defined as a measure of the degree of damage to hosts caused by organisms, and its extent mainly depends on the ability of pathogens to replicate within the host, and may be influenced by multiple factors such as the number of infecting organisms, the route of invasion into the body and tissue tropisms (Casadevall & Pirofski 2001; Peterson 1996). In many experiments assessing the pathogens' capacity to cause the death, lesions or

illness, virulence can be determined by calculating the number of viruses required to cause animal death or disease symptoms in a set period of time following designated routes. The virulence of different organisms is then compared, based on a lethal dose killing 50 percent of experimental animals (LD_{50}) or an effective dose causing disease manifestations in 50 percent of experimental animals (ED_{50}). Pathogenicity, on the other hand, is better thought of as the ability of a pathogenic agent to cause disease or damage in the host. Pathogenicity is therefore determined by a combination of factors involving the host and the environment, which lead to the pathogenicity of an organism varying in different conditions and host species. Understanding virulence and pathogenicity as host-pathogen interaction rather than independent determinants of pathogenicity may provide better insights into the underlying mechanism of pathogenicity caused by pathogens (Casadevall & Pirofski 2001).

Before the development of molecular tests and sequencing, Health Organisation protocols used to assess NDV pathogenicity ranged from evaluation in eggs to those in adult birds. Evaluations of pathogenicity include Mean Death Time (MDT) in chicken embryonated eggs, the intravenous pathogenicity index (IVPI) in six-week-old chickens, and intracerebral pathogenicity index (ICPI) in one-day-old SPF birds (OIE 2018). However, due to the lack of sufficient reliability and accuracy of MDT and IVPI tests for assessing NDV virulence, ICPI test in general is considered to be a better indication of virulence of ND viruses (Dortmans, Jos CFM et al. 2011). The clinical weighed score of ICPI ranges from 0.0 to 2.0 and ICPI value ≥ 0.7 classifies a strain as virulent. Typically, mesogenic strains have the ICPI values ranging from 0.7 to 1.5, and ICPI values over 1.5 indicate velogenic strains (OIE 2018; Swayne & Glisson 2013).

1.2.5 Molecular determinants of NDV-GVII virulence

The amino acid sequence of the F protein cleavage site and the ability of cellular proteases to cleave F protein are a key determinant of NDV virulence (Nagai, Y. 1995; Nagai, Yoshiyuki,

Klenk & Rott 1976; Peeters, BP et al. 1999). The precursor glycoprotein F0 must be cleaved to F1 and F2 to generate infectious viral particles. Cleavage of F0 is carried out by host cell proteases and which proteases cleave is determined by the cleavage site sequence (Nagai, Y. 1995; Rott & Klenk 1988). The consensus sequence of the F protein cleavage site in lentogenic NDV strains is ^{112}(G/E)(K/R)Q(G/E)RL^{117}, whereas in velogenic and mesogenic strains it is ^{112}(R/K)R/Q(R/K)RF^{117} (Samal et al. 2011). Thus, virulent strains have a multi-basic amino acid cleavage site that can be cleaved intracellularly by host proteases found in a wide range of tissues and organs, triggering fatal systemic infectivity. In contrast, the cleavage site of avirulent strains is a mono-basic amino acid motif and can be cleaved extracellularly by trypsin-like enzymes found in the digestive and respiratory tracts, limiting viruses to replicate in these organs, causing mild gastrointestinal and respiratory disease (Nagai, Yoshiyuki, Klenk & Rott 1976; Suarez et al. 2020). This suggests that NDV pathogenicity is primarily determined by the sequence at the F protein cleavage site, but the degree of virulence among many virulent and avirulent strains remains unknown. Even though the F gene is generally accepted to be a major determinant of NDV virulence (de Leeuw et al. 2003b), the successful fusion process for paramyxovirus always requires cooperation of both the F protein cleavage and HN protein attachment (Deng et al. 1997; Gravel & Morrison 2003; Stone-Hulslander & Morrison 1997).

The HN protein of NDV is a multifunctional protein that plays a crucial role in the process of infection. It is not only responsible for mediating the attachment of virus particles to sialic acid-containing receptors on the host cell surfaces, but also promotes the F protein fusion activities, allowing the virus to penetrate the cell surface (de Leeuw et al. 2005b; Morrison 2003). It also acts as a neuraminidase by removing sialic acid from progeny virus particles to prevent self-agglutination during budding (Gravel & Morrison 2003). Moreover, the homolytic interaction with F protein is necessary to drive viral replication, especially in the

tissue tropism and pathogenicity of virulent NDVs (Cornax, Ingrid et al. 2013; Kim, S-H et al. 2011). According to Huang *et al.*, reciprocal HN gene replacement between lentogenic and virulent NDV strains demonstrated that the stem region and global head of HN protein are involved in determining the tissue tropism and virulence (Huang et al. 2004). Nevertheless, viral pathogenicity was not enhanced by the replacement of only the HN gene of a mesogenic virus with the HN gene of neurotropic and viscerotropic velogenic viruses (Estevez et al. 2007). More recent studies have focussed on the combined roles of NDV genes in virulence. Exchanges of single envelope-associated protein genes (M, F and HN) as well as combination of F and HN genes of NDV-GVIId with NDV-GIV, failed to cause spleenoic necrosis (Kai et al. 2015). However, simultaneous inclusion of homologous M, F and HN genes produced severe necrotic lesions and lymphocyte depletion (Kai et al. 2015). Although a number of independent studies have been carried out on the virulent role of viral envelope proteins, it is still difficult to be clear on what really matters. For example, the replacement of both F and HN of velogenic viruses of CA02 and TkND strains with mesogenic virus of Ahinga strain did not produce any changes in virulence (Estevez et al. 2007), while the virulence was altered from velogenic or lentogenic to mesogenic when the exchange of envelope-associated proteins between NDV-GVII S10 strain and La Sota strain were performed (Yu et al. 2017). These studies suggest that the virulence of NDV is affected by additional factors.

The NP, P and L proteins forming the ribonucleoprotein complex are internal proteins that make a substantial contribution to NDV virulence. Similar to studies on the envelope-associated proteins, essential roles of polymerase-associated proteins in virulence have also been observed using reverse genetics. L significantly contributes to NDV virulence, while the roles of NP and P are inconsistent (Dortmans, J. C. et al. 2010; Rout & Samal 2008). In other research on the contribution of individual viral genes to NDV virulence and

pathogenesis, it is illustrated that F protein was the major single contributor, followed by the polymerase L protein (Paldurai et al. 2014; Yu et al. 2017). Moreover, these studies also indicated that the envelope-associated proteins have a greater role than polymerase-associated proteins, and the activities of the internal proteins were directly correlated with viral RNA transcription and replication.

The relationship between NDV replication and levels of virulence in virus infection has also been studied. V protein in particular was proved to be essential for NDV replication and serves as a virulence factor. The role of V protein in moderating NDV virulence involves a mechanism that degrades phospho-STAT1 to block type I interferon signalling (Alamares et al. 2010; Huang et al. 2003). NDV pathogenicity in chicken embryos was markedly attenuated by low expression of V protein in recombinant NDV, while the overexpression of V protein can significantly reduce the host cellular innate immune system and increase virus replication (Jang et al. 2010; Mebatsion et al. 2001). This protein functions as an IFN antagonist that allows NDV to evade the host innate immune response by inhibiting type I and II interferon and apoptosis responses in NDV infection (Huang et al. 2003; Wang, C et al. 2018). IFN-antagonistic activities of V proteins from different strains are quite diverse, driven by amino acid differences in the C-terminal and N-terminal domains of V proteins, and are associated with their virulence (Alamares et al. 2010; Wang, X, Dang & Yang 2019). Additionally, V protein is a determinant of host range restriction via its species-specific IFN antagonist activity (Park, M-S et al. 2003). V is also a multifunctional protein and plays an important role in NDV replication and survival in the host. In summary, virulence of NDV is a complex trait determined by multiple genes, with the multiple basic amino acid sequence motif of F_0 precursor being the minimum prerequisite of virulence.

1.3 Lymphotropic pathology of virulent NDV

NDV is rapidly evolving, and several highly pathogenic genotypes have been uncovered over the past 20 years. NDVs vary substantially in their virulence/pathogenicity, and based on pathotypes they can be divided into velogenic, mesogenic and lentogenic isolates (Alexander, D 2000). The severity of disease caused by NDV infection depends on multiple factors related to host species, age, the route of exposure, immune status and field conditions, but the virulence of the infecting strain is the most important factor. While lentogenic and mesogenic NDV strains frequently cause mild signs or unclear disease in the gastrointestinal tract and respiratory system, highly virulent NDV viruses tend to produce deadly acute infections with profound clinical symptoms and intensive haemorrhagic lesions in the lymphoid tissues (Brown, C, King & Seal 1999; Ezema et al. 2016; Piacenti et al. 2006). Chickens and turkeys are the most sensitive species to virulent NDV from an outbreak in 2002 to 2003 in California, whereas pigeons are the least susceptible and are capable of carrying this highly virulent NDV strain without clinical signs (Kapczynski, D. R. & King 2005).

It is well-established that the amino acid sequence at the F protein cleavage site is the major molecular determinant of NDV virulence (de Leeuw et al. 2003a; Peeters, BP et al. 1999). However, more recent studies revealed that despite sharing a similar F cleavage site and high ICPI values associated with severe virulence, virulent strains of distinct genotypes of NDV induce different pathogenic outcomes in diseased birds, especially in lymphoid organs, as shown Table 1.2 (Brown, C, King & Seal 1999; Ecco et al. 2011; Ezema et al. 2016; Hu, Zenglei et al. 2015; Piacenti et al. 2006; Susta et al. 2011).

Table 1.2. The severity of lesions between virulent NDV strains of different genotypes in tissues

Affected tissue	G-I (Australia strain)	G-V (Nevada cormorant strain)	G-VII (Long Bien strain)	G-IV (Herts/33 strain)	G-IX (F48E8 strain)
Spleen	+	+	++++	+	++
Thymus	-	-	+++	+	+
Bursa	-	-	+++	+	+
Cecal tonsil	-	+	+++		
Eyelid	+	+	+++		
Pancreas	-	-	++		
Lung	+	-	-		
Heart	-	+	-		
Brain	-	+++	-		
Bone marrow	-	-	+++		

++++ severe lymphocyte depletion, histiocytosis, and extensive necrosis (>50%); +++ moderate lymphocytic depletion, histiocytic accumulation and necrosis; ++ mild lymphocyte depletion; + mild hyperplasia of lymphocytes; - normal.

Particularly, NDV-GVII Long Bien strain produces more severe lesions in lymphoid tissues than NDV-GI Australia and NDV-GV Nevada cormorant strains (Harrison et al. 2011; Susta et al. 2011). Ecco *et al.* has revealed that virulent NDV genotype V- CA02 and genotype VII-rZJ1 strains cause more severe clinical signs and pathogenic changes in the spleen compared to the other velogenic strains (turkey ND, Australia and Texas GB/48 strains) (Ecco et al. 2011). The severity of signs and pathological outcomes can also be different within the same velogenic NDV genotype, especially when observing the pathogenicity of genotype V-NDV strains. The severe clinical signs and microscopic lesions in the lymphoid tissues caused by NDV genotype V-Quail2006 strain are more severe than those caused by NDV genotype V-Chicken2000 isolate (Merino et al. 2011). These findings indicate that clinicopathological manifestation caused by the virus must be determined by additional factors. The severity of the clinical signs and the pathogenesis of virulent NDV is associated with the strong innate

immune response of the host to the infection. Virulent NDV-GVII strains cause strong innate immune and intensive inflammatory response in lymphoid tissues compared to other virulent NDV strains such as GIV, GVIII and GIX genotypes, by activating a group of genes regulating inflammatory and innate antiviral responses (Hu, Z. et al. 2015; Rasoli et al. 2014). These studies also revealed that the extent of the host innate immune response is mainly determined by NDV virulence. As mentioned previously, the different pathological outcomes are dependent on velogenic NDV strains of distinct pathotypes. NDV-GVII strains, which are currently endemic in several countries, especially in Southeast Asia, (Doan et al. 2020; Miller et al. 2015; Xiao, S. et al. 2012b), and produce more severe damage in the lymphoid tissues compared to virulent strains of other genotypes characterised by severe lymphocyte depletion and necrosis of the spleen, bursa and thymus (Adi et al. 2010; Harrison et al. 2011; Kommers, G et al. 2002). It appears that the efficiency of viral replication in target tissues determines the magnitude of host innate immune response. Virulent NDV-GVII strains replicate at substantially higher levels in lymphoid organs than other virulent NDVs, release more viral particles, and activate the pattern recognition receptors that are associated with strong innate immune and intense inflammatory responses (Hu, Z. et al. 2015; Rasoli et al. 2014; Xiang et al. 2018). Furthermore, velogenic NDV pathogenesis can be influenced by host innate immune response through its interaction with apoptosis and necrosis. NDV infection directly induces apoptosis leading to histological changes, including severe tissue damage and necrosis in the lymphoid tissues (Harrison et al. 2011; Kommers, G et al. 2002; Kommers, GD et al. 2003). Virulent NDV-GVII strains induce substantial levels of apoptosis in lymphoid tissues during early infection that directly contributes to lymphocyte depletion and the death of infected birds, compared to other virulent NDV strains (Ecco et al. 2011; Kommers, GD et al. 2003; Wang, Y et al. 2012). These studies suggest that the apoptosis and severe necrosis in lymphoid tissues, caused by NDV-GVII, could contribute to lymphocyte

depletion, which is strongly associated with the severity of the clinical outcomes. By focusing on the role of cell death in the pathogenesis of NDV-GVII, our recently published paper characterised mRNA profiling using RNA-seq , revealing that NDV-GVII infection induces the activation of autophagy-related cell death as well as lymphotropic and synaptogenesis signalling pathways, suggesting the involvement of autophagy in NDV-GVII pathology (Rabiei et al. 2021). Taken together, the destructive effects on the immune system caused by the switch to lymphotropic behaviour may be an indispensable part of virulent NDV pathogenesis. However, the molecular pathogenesis and interaction between the host innate immune response and virulent NDV-GVII need further study.

1.4 Host immune response to NDV infection

The innate immune response is a first line of defence prior to infection and is capable of eliminating or inhibiting pathogen invasion and replication. There are four main components of natural immunity in chickens: physical and chemical defences such as the skin, and mucosa; phagocytosis by natural killer cells, macrophages and other phagocytic cells; inflammatory mediators and complement proteins; and cytokines and chemokines. Generally, the host innate immune response against viral infection is designed not only to control or prevent viral replication and spread, but also to alert the adaptive immune system. Like other viral infections, single stranded NDV RNA is detected by the host innate immune system through pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs), the nucleotide oligomerization domain-like receptors (NLRs), RNA helicases such as the retinoic acid-inducible gene 1-like receptors (RLRs) or MDA5, and C-type lectins. The intracellular signalling cascades induced by PRRs lead to transcriptional expression of inflammatory mediators that coordinate to eliminate pathogens and infected cells. The impact of NDV on the host has been examined in a wide range of studies to provide better understanding of the transcriptional host response of chickens and the

pathogenesis of NDV strains using multiple techniques. The most frequent method is to examine mRNA expression of key immune-related genes by qRT-PCR. In egg-laying hens challenged with virulent NDV-GVII, a significant upregulation of TLR3/7/21, MDA5, IL-2/6/1β, IFN-β, CXCLi1/2, and CCR5 in the magnum and uterus occurs (Li, R et al. 2016). Gene expression profiles in the spleen showed an up-regulation of CCLi3, CXCLi1, CXCLi2 (IL-8), IFN- α, IL-12α, IL-18, IL-1β, IL-6, iNOS, TLR7, MHCI, IL-17F and TNFSF13B after challenge with velogenic NDV (Rasoli et al. 2014). While the majority of these studies revealed that NDV strains with different virulence result in distinct host cytokine expression patterns, velogenic NDV strains show higher replicability and induce stronger innate immune response (Baier, Pfeifhofer & Thuille 2012). For example, although both NDV-GVII and La Sota can infect mature chicken bone-marrow derived Dendritic Cells, immune-related genes including MDA5, LGP2, TLR3, TLR7, IFN-α, IFN-β, IFN-γ, IL-1β, IL-6, IL-18, IL-8, CCL5, IL-10, IL-12, MHC-I, and MHC-II levels were significantly upregulated in challenge with NDV-GVII compared to La Sota (Xiang et al. 2018).

Prior to the advent of RNA-seq, microarray analysis was used to analyse transcription in chickens infected with NDV. Microarray experiments were performed on spleens from SPF chickens after challenge with virulent NDV and host gene expression profiles were examined at day 1 and 2 (Rue et al. 2011), revealing that virulent NDV induced a robust innate immune response after infection with the induction of important genes involving in pro-inflammatory and antiviral cytokine responses including IL6, MIP-3α, Mx, lysozyme, IFIT5, (ISG)12-2, MDA5 at 1 dpi and IFNγ, iNOS, IL-1β, IL18, IL8, PKR, OAS, K203, ah221 at 2 dpi (Rue et al. 2011). Furthermore, Hu *et al.* indicated that virulent NDV-GVII caused a much higher cytokine response in lymphoid tissues such as spleen and thymus compared to other virulent NDV strains and was associated with lymphocyte destruction (Hu, Z. et al. 2015). These cytokine genes were significantly enriched for functions of inflammatory response, cell

death, tissue morphology, and haematological system development and function. However, a study on host cell transcription associated with pathogenic NDV infection showed that virulent NDV infection not only triggers an inhibitory effect on interferon-stimulated genes, but also causes downregulation of cytoskeletal proteins involved in virus entry, replication and transport, and increase in apoptotic-mediating thioredoxin protein (Munir, Sharma & Kapur 2005).

The introduction of RNA-seq allowed detection of more Differentially Expressed Genes (DEGs) with higher fold change, the identification of genetic variants, and the elimination of technical issues associated with microarrays affecting data accuracy (Zhao, S et al. 2014). An in vitro study analysing transcriptome profiles of chick embryo fibroblasts infected with highly pathogenic NDV using RNA-seq found 8,433 transcripts that were differential expressed after NDV infection, of which 3,616 were interferon-stimulated genes (ISGs) enriched in well-known pathways such as the actin cytoskeleton, MAPK signalling pathway, protein processing in endoplasmic reticulum and focal adhesion (Liu, W et al. 2018). Similarly, (Zhang, Jibin et al. 2018) revealed that ISGs were found as important DEGs in regulating immune responses to the avirulent La Sota strain in chickens in vivo. Transcriptome analysis of spleens also indicated that most differentially expressed genes were grouped into signalling pathways including EIF- signalling, actin cytoskeleton organisation, nitric oxide production, and coagulation systems that were associated with resistance to NDV in chickens (Zhang, Jibin et al. 2018). Despite the increasingly common use of RNA-seq in chickens, to the best of the author's knowledge, host-pathogen interplay of birds infected with newly emerged virulent NDV-GVII in vivo using RNA-seq was first addressed by our group. In our recently published paper, roles of apoptosis and necrosis in disease severity and tissue damage were characterised (Rabiei et al. 2021).

It is obvious that highly pathogenic NDV strains trigger a much stronger innate immune response than avirulent NDVs. However, this substantially higher level of innate immune response does not prevent morbidity and mortality due to velogenic NDV, and more importantly, it could be destructive to host lymphoid tissues (Rue et al. 2011). Although the host innate immune system is critical for containing and controlling pathogen infection, alone it is not enough to ensure the host survival after an exposure to a velogenic NDV-GVII. Hence, once the virus overcomes innate and cellular immune responses, humoral immunity is required.

1.5 Virulent NDV and vaccination

Vaccination has a very important role in preventing losses from morbidity and mortality. Although different genotypes of NDV display genetic and antigenic divergence, all NDV strains belong to one serotype, which has allowed lentogenic isolates such as La Sota, B1, Ulster, VG/GA and V4 to be used as inactivated and attenuated vaccines worldwide over decades (Miller et al. 2013; Suarez et al. 2020). In contrast, the application of mesogenic vaccine velogenic strains of other genotypes including genotype II, III, Vb, & VIId is very limited (Suarez et al. 2020). The lentogenic live-attenuated La Sota and B1 vaccines are the most commonly used in many countries and can provide 100% protection against virulent NDV if the vaccine quality is good enough, correctly administered to healthy chickens and high levels of antibody titters are reached prior to exposure to virulent virus (Cornax, I., Miller & Afonso 2012; Mahmoud et al. 2019; Miller et al. 2013; Roohani 2015). Unfortunately, recent ND outbreaks have occurred in heavily vaccinated flocks, and highly virulent NDVs have been isolated from these vaccinated birds, indicating that these field variants escape vaccine antibodies (Doan et al. 2020; Dortmans, Jos CFM, Peeters & Koch 2012). The failure of vaccines may be due to poor flock immunity resulting from inadequate vaccination practices, such as insufficient dosage and/or improper scheduling, not vaccinating

the whole flock, or from vaccine mismatching with field strains. Avirulent vaccine strains cannot confer full protection against virulent NDV-GVII (Dortmans, J et al. 2014; Roohani 2015). Lentogenic ND vaccines derived from genotype II can protect chickens from NDV-II induced mortality, but they do not fully confer protection against infection and viral shedding infection by strains from other genotypes, leading to continuous spread of field virus to vaccinated birds (Kapczynski, Darrell R, Afonso & Miller 2013; Miller et al. 2007; Roohani 2015; Shahar et al. 2018). These findings highlight an essential need for antigenic-matched NDV-GVII vaccine to improve vaccine efficacy in chickens against NDV-GVII field strains.

1.6 Transcriptomic analysis

The transcriptome is the key intermediate between the genome and the proteome and consists of all RNA molecules in a cell. Information on how an organism's genes are regulated, and the details of its biology are partly revealed by measuring the expression of these genes in different tissues, time points or conditions. In addition, the functions of previously uncharacterised genes also inferred through this approach. Understanding the transcriptome enables researchers to elucidate the functional elements of the genome and reveals the molecular constituents of tissues and cells as well as helping to understand factors that may affect the onset and development of disease. In general, transcriptomics is a powerful and useful technique allowing researchers to identify and measure changing expression levels of each gene/transcript under different conditions or during development. Moreover, transcriptome profiling can empower cataloguing all species of transcripts such as mRNAs, small RNAs and non-coding RNAs. It also aims to determine the transcriptional structure of genes: their start sites, splicing patterns and other post transcriptional modifications (Lowe et al. 2017).

Early approaches to study whole transcriptomes used hybridization or microarray methods that have several limitations such as lack of accuracy, sensitivity or high costs, whereas

methods such as Northern blotting and QRT-PCR can only capture a small fraction of a transcriptome and are relatively laborious. In contract, RNA-seq is a technology that has revolutionized RNA biology over the past ten years. Transcriptomes of hundreds of individuals can be sequenced at once. Additionally, while RNA-seq requires low sample input, the number of detected transcripts increases linearly with increasing sample volume, is not limited by the technology and has low background noise, enabling highly accurate quantification of expression levels (Wang, Z, Gerstein & Snyder 2009). A basic RNA-seq workflow is illustrated in Fig 1.3, including three main parts.

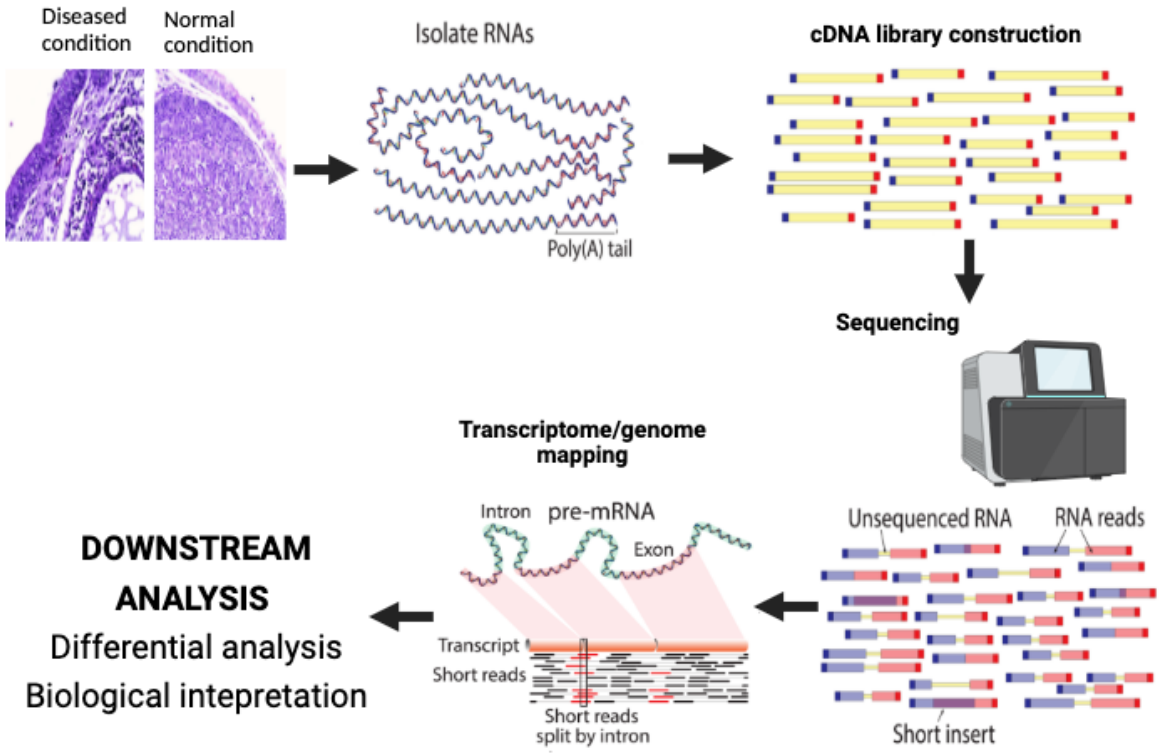


Fig 1.3 RNA-seq workflow

The first section consists of RNA preparation, cDNA library construction and sequencing. Total RNAs is isolated from tissue samples and genomic DNA were removed. It is then checked for quality, purity, and integrity before converting into cDNA libraries. The library

construction and sequencing mainly depend on sequencing platforms and the kits that are used. Different RNA-seq machines and platforms have their own technical specifications such as costs, run time, error rates, sequence yields, etc (Han et al. 2015). Library construction requires Nucleic Acid prepares samples in a way that is compatible with the sequencer, including DNA fragmentation, adaptor addition etc. In order to save resources, multiple libraries can be barcoded, pooled and sequenced in the same run (a multiplexing process) (Han et al. 2015; Wolf 2013). For sequencing, libraries are loaded onto a flow cell and placed on the sequencer. Fluorescently labelled nucleotides are added in cycles and are recorded for their fluorescent signal.

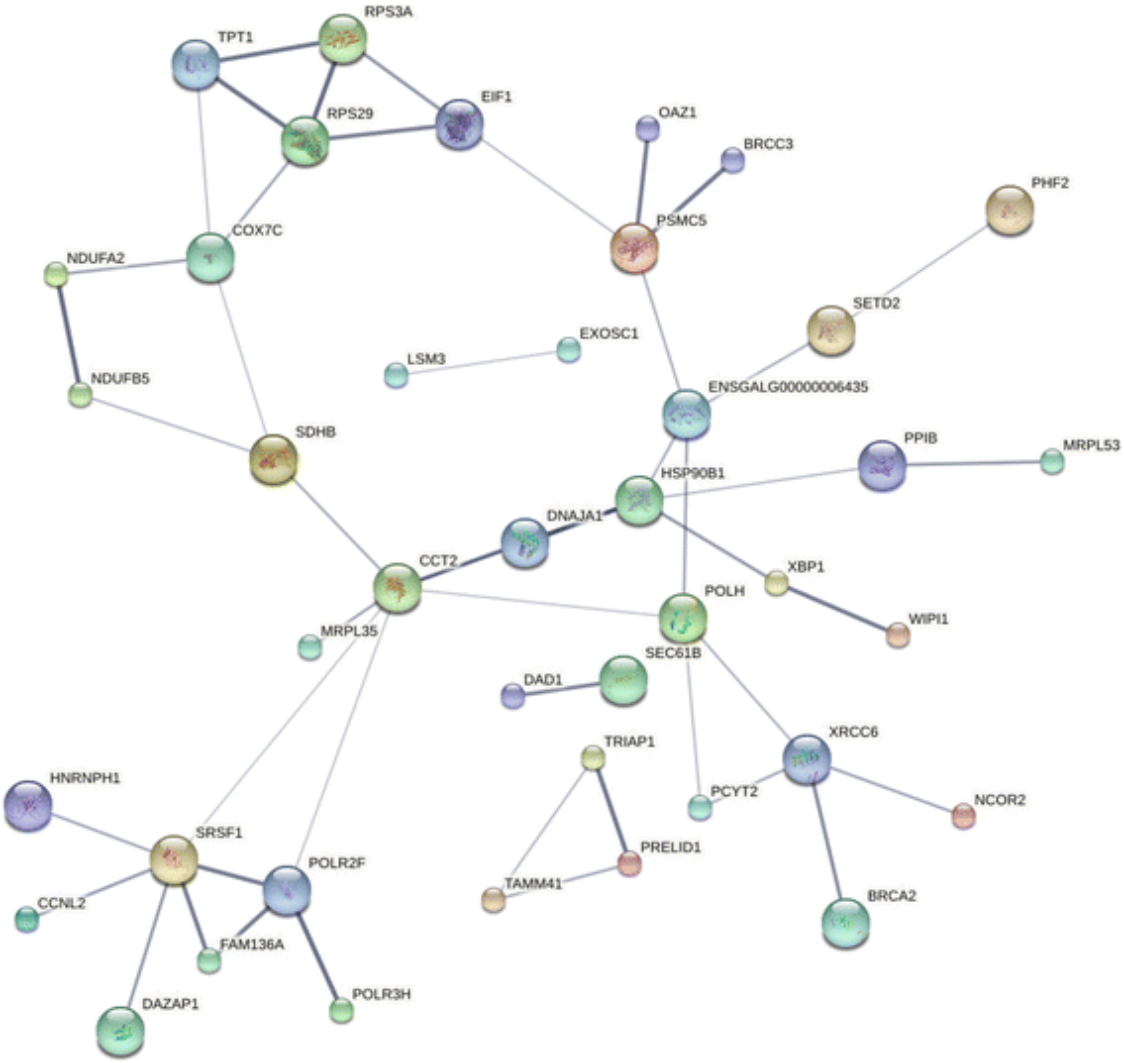
The second part of an RNA-seq workflow is the computational biology, starting with raw reads as an input. In brief, reads from each sample are subjected to quality assessment, adaptors removed, and usually mapped to a reference genome (although reference free methods are also available). The number of reads mapping to each gene are counted, and data normalized. A number of tools, programs and packages are available for these steps (Han et al. 2015). The final step of RNA-seq workflow is downstream analysis allowing identification of DGEs, interpretation of the biological context, and uncovering the molecular components that help shed light on the molecular mechanism of the development and progression of disease.

Generating a list of DGEs is the first step to gaining a biological understanding of developmental stages, molecular mechanisms of diseases or experimental systems. To provide further insights into the biological context of DGEs, pathway enrichment analyses are usually performed. Enriched function analyses mainly rely on the use of knowledge databases such as Enrichr (Kuleshov et al. 2016), ClueGO and CluePedia (Bindea, Galon & Mlecnik 2013; Bindea et al. 2009), GO (Ashburner et al. 2000), KEGG: Kyoto encyclopedia of genes and genomes (Kanehisa & Goto 2000), DAVID(Dennis et al. 2003) and other

commercial annotation database systems such as Pathway Studio or Ingenuity Pathway Analysis (IPA). However, the molecular basis of disease cannot be uncovered only by functional enrichment pathway analysis. These pathways basically describe signalling, neural, metabolic and genetic processes as a serial sequence of biochemical reactions where substrates are altered in a linear chain. In contrast, protein-protein interaction networks map functional or physical interactions between gene/protein pairs generating a complex grid of connections. Additionally, disease genes tend to group together and co-express as central network loci, and proteins involved in similar phenotypes are highly interconnected. Hence, protein-protein interaction networks can be used to identify novel pathways and gene networks and provide better insights into disease (Gonzalez & Kann 2012).

1.7 Protein-protein interaction (PPI) networks and disease

Major biological processes are regulated by protein interactions. PPI represents interactions between two or more proteins coming together to complete biological processes, in which genes/proteins and interaction between them act as nodes and edges, respectively (Fig 1.4) (Deist, Melissa S. et al. 2017). Proteins do not function individually; they mostly interact with other proteins or with other molecules such as RNA and DNA, regulating cellular processes, signalling and metabolic pathways (Kann 2007).



582
583 Fig 1.4 The gene interaction network impacted by avirulent NDV challenge and line, in
584 which genes and interaction among them represent as nodes and edges, respectively.

585 Recent advances in high-throughput sequencing technologies, bioinformatics tools and public
586 databases have empowered researchers to characterise molecular changes and differences
587 between healthy and diseased organisms, leading to an increasing amount of disease related
588 genes identified based on new protein interactomes (Ideker & Sharan 2008). This also means
589 that functions of previously unknown proteins can be predicted from PPI networks through
590 their role in protein complexes or pathways. Proteins have a central role in biological
591 function and their interactions determine the molecular and cellular mechanisms that control
592 healthy and diseased states in organisms, whereas diseases are often a result of mutations that

cause disorders of the protein-binding interface or biochemically dysfunctional allosteric changes in proteins (Sevimoglu, Tuba & Arga, Kazim Yalcin 2014). Therefore, such networks provide a better understanding of the pathogenic mechanisms that cause the progression and onset of disease. In genetic diseases research, one main focus is to identify causal genes from the molecular structure. The same disease genes are highly physically interconnected via the proteins they encode and indirectly interact with each other. In addition, the potentially causative genes of some complex diseases are likely to be located within the same network groups such as subnetworks of a given biological network or biological modules or protein pathways (Safari-Alighiarloo et al. 2014). PPI networks have been used in several previous studies to be able to understand gene and protein interaction (Masood, Manjula & Sugumaran 2018; Zhang, J. et al. 2013; Zhang, J. et al. 2016). The promising applications of PPI networks to complex disease are: to identify novel disease genes and proteins; identify disease-related subnetworks; study network properties; and classify network-based disease genes (Kann 2007; Sevimoglu, T. & Arga, K. Y. 2014). A PPI network is constructed using a dataset containing interacting protein pairs and an interaction score retrieved from the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (Szklarczyk et al. 2019). The network is generated with genes/proteins as nodes and the edge between nodes joined if there is a significant co-expression relationship between them. Computational approaches such as Cytoscape (Shannon et al. 2003) are then used to manipulate and interpret such interaction networks for further analysis such GO enrichment and KEGG pathway analysis. Through GO and KEGG analysis of genes, novel disease genes can be validated and significantly enriched with GO and KEGG terms that indicate their possibly indirect or direct relationships with the origin or progression of a particular disease (Masood, Manjula & Sugumaran 2018). Hence, studies on

PPI network can decipher the molecular details of the processes leading to diseases, which in turn can contribute to methods for diagnosis, prevention, and treatment.

CHAPTER 2: GENOME SEQUENCES OF NEWCASTLE DISEASE VIRUS STRAINS FROM TWO RECENT NEWCASTLE DISEASE OUTBREAKS

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Statement of Authorship

Title of Paper	**Genome sequences of Newcastle disease virus trains from two outbreaks in Indonesia**
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Principal Author

Name of Principal Author (Candidate)	Phuong Thi Kim Doan		
Contribution to the Paper	Performed laboratory work, analysed, and interpreted data, wrote manuscript and act as corresponding author		
Overall percentage (%)	65%		
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	09/12/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	16.12.2021

Abstract: The genomes of two newly emerged Newcastle disease viruses, Chicken/Indonesia/Mega/001WJ/2013 and Chicken/Indonesia/Cimanglid/002WJ/2015, are reported from disease outbreaks in chickens in Indonesia. Phylogenetic analysis of different genotypes of Newcastle disease virus using the F gene coding sequences suggests that these two strains belong to genotype VII.2, class II of avian paramyxoviruses. They were closely correlated with the chicken/Sukorejo/019/10 strain that caused outbreaks during 2009-2010. Amino acid identities of all genes vary between 81 – 94% in comparison with the La Sota vaccine strain.

2.1 Introduction

Newcastle disease is one of the most severe infectious diseases of chickens. The causative agent, Newcastle disease virus (NDV), is a member of the Avian genus *Orthoavulavirus*, subfamily *Avulavirinae*, family *Paramyxoviridae* (ICTV 2019). The poultry industry in South-East Asia and Middle East in recent years has severely devastated by recurrent Newcastle disease virus (NDV) outbreaks (Dimitrov, K. M. et al. 2016). The devastating impacts of ND are not only very high mortality but also economic losses from embargoes and trade restrictions (Alexander, D 2000). NDV is capable of infecting over 240 species of birds mainly through direct contact between healthy and diseased birds (Alexander, D.J & Jones 2008; Miller, Decanini & Afonso 2010).

NDV was first reported in Java, Indonesia in 1926 and then spread to the rest of the world. It is a single-stranded, non-segmented, negative-sense and enveloped RNA virus with 6 major structural proteins in the order 3'-NP-P-M-F-HN-L-5'(Cornax, Ingrid et al. 2013; Xiao, S. et al. 2012b). Base on the clinical signs displayed in infected birds, NDV strains can be divided into five pathotypes (Alexander, D 2000): (1) viscerotropic velogenic viruses causing sudden deaths of chickens with haemorrhagic lesions in the gastrointestinal tracts of infected birds; (2) neurotropic velogenic viruses responsible for high mortality with respiratory and

neurological symptoms; (3) mesogenic viruses producing low mortality with the signs of respiratory and neurological disease; (4) lentogenic strains causing mild infections of the respiratory disease and are asymptomatic in the intestinal tract; (5) avirulent viruses replicating in the intestine with no clinical symptoms, mainly use as live vaccines. Based on the phylogenetic analysis of the F (Fusion) and HN (Haemagglutinin-Neuraminidase) proteins of NDV isolates, NDV strains can be separated into two classes; class I virus includes a single genotype with 3 sub-genotypes that have been isolated mainly from wild birds and are usually avirulent, whereas class II virus has 21 genotypes (I-XXI) that have been recovered from wild and domestic birds and consist of both virulent and non-virulent strains (Dimitrov et al. 2019; Miller, Decanini & Afonso 2010; Orynbayev et al. 2018). Based on the phylogenetic analysis of the F (Fusion) and HN (Haemagglutinin-Neuraminidase) proteins of NDV isolates, NDV strains can be separated into two classes; class I virus includes a single genotype with 3 sub-genotypes that have been isolated mainly from wild birds and are usually avirulent, whereas class II virus has 21 genotypes (I-XXI) that have been recovered from wild and domestic birds and consist of both virulent and non-virulent strains (Dimitrov et al. 2019; Miller, Decanini & Afonso 2010; Orynbayev et al. 2018).

In order to control NDV, the poultry farms have heavily relied on vaccination, but Newcastle disease is still major thread for poultry industry worldwide (Adi et al. 2010; Dimitrov, Kiril M, Lee, Dong-Hun, et al. 2016), questioning the efficiency of the vaccination strategy widely used throughout continents, especially Indonesia where the ND serious problem is as an endemic infection. Indonesian Veterinary drugs index showed that the distribution of NDV vaccine is very diverse using various ND vaccine seed types as shown in Fig 2.1

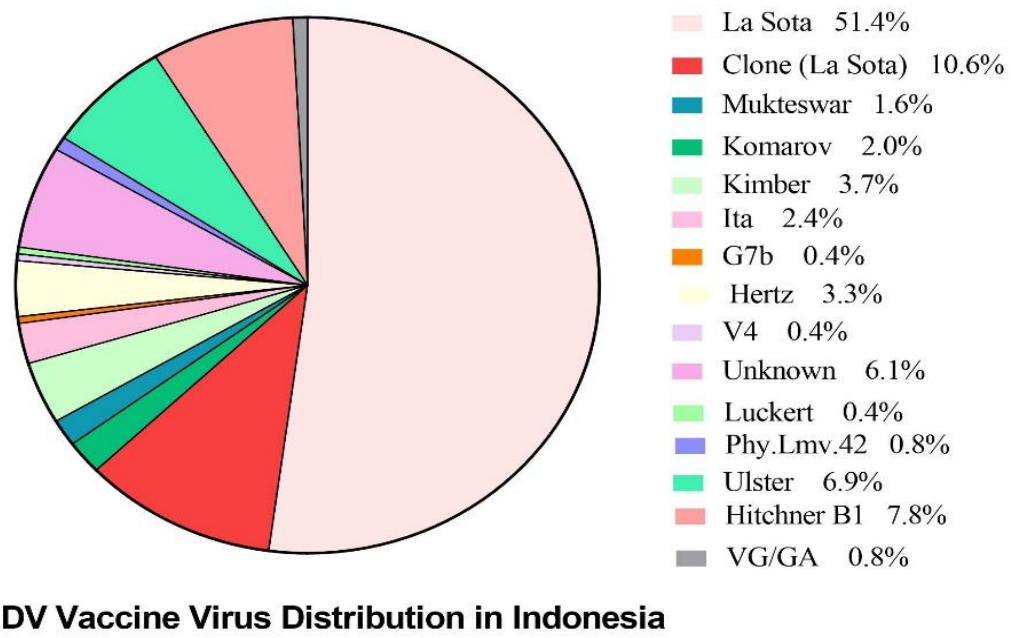


Fig 2.1. ND vaccine virus distribution in Indonesia (source: Indonesia Veterinary Drugs Index)

As shown in Fig 2.1, 51,4% of ND vaccine seeds are La Sota strain and used by most of the ND vaccine distributors, followed by Clone (La Sota) with 10,6% and Hitchner B1 with 7,8%. Only 0.4% of vaccine seeds is G7b strain that is produced in Indonesia. Meanwhile, many recent major ND outbreaks in Indonesia, Southeast Asia have appeared in commercial chickens, leading to 70-80% mortality and have been caused by the virulent NDV genotype VII (NDV-GVII) according to recent phylogenetic analysis of amino acid sequence of F and HN proteins (Berhanu et al. 2010; Nlp et al. 2014; Xiao, S. et al. 2012b). Many studies also speculated that the fourth and fifth ND pandemics in Africa, Europe, Middle East and Asia have been associated with the NDV-GVII (Dimitrov et al. 2019; Miller et al. 2015). Furthermore, amino acid identity matrices of the F and HN proteins obtained from some isolates in Indonesia have been compared to B1 and La Sota vaccine strains. The similarities of identity matrix between the Indonesian strains and the vaccine strains B1 and La Sota ranging from 87% to 89% were significant, which indicate that the vaccine strains were substantially distinct from the circulating strains in Indonesia. As a consequence of antigenic

differences, the vaccine protection is poor in the NDV-GVII outbreaks (Xiao, Sa et al. 2012a). Determining the most frequently circulating NDV strains in intensive poultry farms using next gene sequencing has an important role in understanding the evolution and genomic diversity of virulent NDV strains. The first aim of this study is, hence, to isolate and analyse whole genome sequencing of circulating NDV strains in vaccinated flocks in Indonesia where there were the high mortality and heavy losses. Moreover, the second aim is to classify the newly emerged NDV viruses and compare their sequences with the types of available ND vaccines in Indonesia.

2.2 Materials and Methods

2.2.1 NDV virus isolation and detection

The two viruses in this study were collected from two brain samples of vaccinated chickens of two different NDV outbreaks in West Java, Indonesia in 2013 and 2015. These samples were processed based on the OIE guidelines for laboratory procedures to isolate the virus (World Organisation For Animal Health 2019), and were then inoculated in 9-day-old specific pathogen-free embryonated chicken eggs and followed by collection of allantoic fluid. In brief, 0,2ml 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. While embryos died in 24 hours were discarded, after 5 days all eggs were chilled at 4°C for five hours. Allantoic fluid from dead embryos (after 72 hours) or after 5 days of incubation was tested in HA test. Haemagglutination positive samples were used for serological and molecular detection of NDV (Choi et al. 2013).

2.2.2 Reverse transcription polymerase chain reaction (RT-PCR)

Viral RNA was isolated from each samples using commercial Viral RNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol. To amplify F gene of each isolate, a F-F1 and F-R1 primer set with product length of 507bp fragment, including the cleavage site was

designed using Primer-Blast. Primers F-F1 and F-R1 were corresponding to nucleotides 278 to 298 and 766 to 784 of the F gene, respectively. Additionally, a primer set of HN-F1 and HN-R1 was designed to amplify complete HN gene of each isolate with a fragment of 1922 base pairs (Ke et al. 2010; Miller et al. 2007). Viral RNA of each sample was subjected to a one step RT-PCR using gene specific primers. In brief, 5µl of extracted RNA was used as a template for a conventional RT-PCR using SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA).

2.2.3 Partial genome sequencing and sequence analysis

The PCR products for both F and HN genes were submitted to Australian Genome Research Facility Ltd (AGRF) for Sanger sequencing. All fragments were sequenced both in forward and reverse directions in order to confirm the correct sequence. To rebuild the whole length sequence, overlapping sequences were edited and assembled using BioEdit v.7.0. Nucleotide sequences of F and HN genes for NDV strains retrieved from Genbank were used as reference viruses for nucleotide alignments using ClustalX (Thompson, Gibson & Higgins 2003), and phylogenetic tree was constructed using Mega v6.0 program.

2.2.4 Pathogenicity index detection

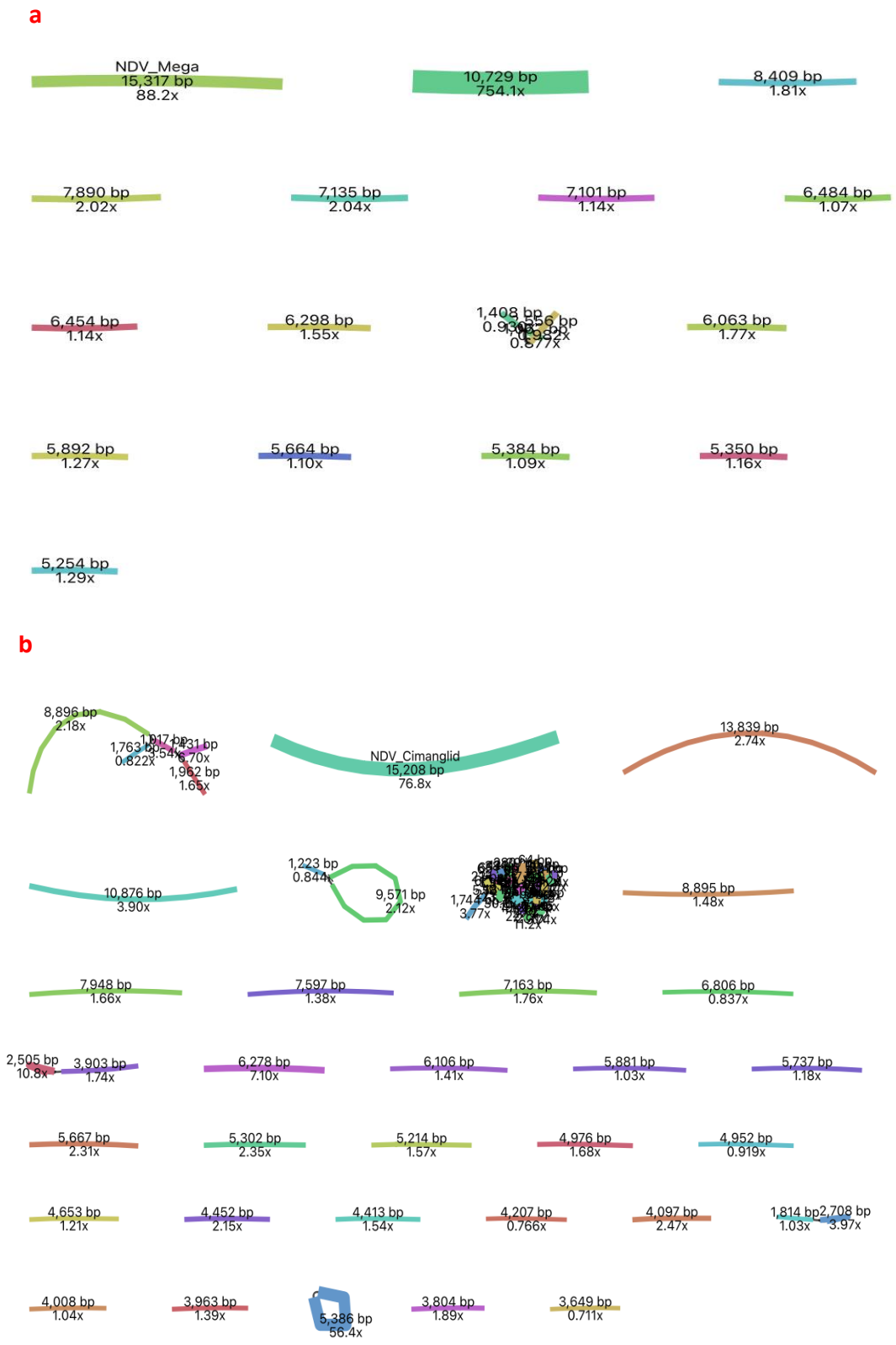
There are two standard methods to determine the virulence of the isolated viruses. In order to determine the Mean Death Time (MDT) for each isolate, a ten-fold serial dilution of infective allantoic fluid of each virus (10^{-6} to 10^{-9}) was prepared in sterile PBS. 100µl of each dilution was inoculated into the allantoic cavity of five 9-day-old embryonated SPF chicken eggs and incubated at 37°C for up to 7 days. The eggs were examined twice a day and the embryo death time was recorded. The lowest virus dilution causing all embryos to die was considered as the minimum lethal dose. According to OIE classification for NDV pathogenesis, velogenic NDV strains has the MDT less than 60 hours, while the MDT for mesogenic strains is between 60 to 90 hours, and for lentogenic strains is more than 90 hours (Grimes

2002; World Organisation For Animal Health 2019). Moreover, ICPI values were measured and reported as 1.58 and 1.50 respectively (out of a maximum value of 2. The virulence of these strains was confirmed by repeating the MDT the same index as measured before (Adi et al. 2010)

2.2.5 Full genome sequencing and sequence analysis

The viral RNA from selected samples were purified using the Viral RNA Mini Kit (Qiagen, USA) and were subjected to full genome sequencing using Next Gene Sequencing. cDNA libraries were made using random hexamers using the KAPA Stranded mRNA-seq Kit (KAPA Biosystems, USA) as per the manufacturer's instructions. The resulting cDNAs were sequenced using Illumina Miseq platform v3 600-cycle kit, generating 2x300 nt reads, and the library size was checked on a Bioanalyzer 2100 using the High Sensitivity DNA kit (Agilent Technologies, Germany). Adaptors were removed using Trimmomatic 0.36 (Bolger, Lohse & Usadel 2014). 616,471 and 794,856 reads for samples 1 and 2, respectively, were de-novo assembled using Unicycler v.0.4.4 with default parameters (Wick et al. 2017) and visualised with Bandage (Wick et al. 2015) and the NDV-Mega and NDV-Cimanglid contigs are shown as Fig 2.2a&b. Assembled contigs were blasted against reference genome (chicken/Sukorejo/019/10 strain, accession number: HQ697255) using NCBI Blast, to identify NDV contigs (Wheeler et al. 2007). Contig 1 of sample 1 and Contig 2 of sample 10 showed the high homology to the NDV reference. They have been named Avian avulavirus 1 chicken/Indonesia/Mega/001WJ/2014 (shorthand Mega/001WJ) and Avian avulavirus 1 chicken/Indonesia/Cimanglid/002WJ/2014 (shorthand Cimanglid/002WJ). Two NDV contigs of 46% genome GC contents and the lengths of 15,317 nt and 15,208 nt were identified for Mega/001WJ and Cimanglid/002WJ, respectively. These contigs had an average coverage of 573x and 1837x and had a 97.97% and 98% identity to the Sukorejo strain (HQ697255), respectively. ClustalX and Bio Editv.7.2 were used to align and edit assembled NDV

sequences. Additionally, Geneious R10.1.3 (https://www.geneious.com) and ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) were used to annotate genes and confirm open reading frames (Wheeler et al. 2007). Interestingly, a gap in the sequence of Mega/001WJ was detected and closed using RT-PCR (QIAGEN) and Sanger sequencing (Sabra et al. 2017). In brief, the set of primer (Mega/001WJ-F: ACCAAACAGAGAATCTGTGAGG and Mega/001WJ-R: CGGATCATCACTGTTAAGGGTG) was designed to amplify a 280bp block of the NDV to be able to cover the UTR and the beginning of Nucleoprotein of Mega/001WJ sequence. The viral RNAs were subjected to a one step RT PCR method and then the PCR product was subcloned into a PMT-Easy vector and finally sequenced using M13 primer pair in both directions and Bio Edit v.7.2 was used to merge sequences (Hall 1999).



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155 Fig 2.2 NDV contigs visualised in Bandage tool. (a. NDV-Mega and b. NDV-Cimanglid).

2.2.6 Sequence and phylogenetic analyses

Sequences for the full length of F and HN genes were aligned with the ClustalW multiple alignment method using Bioedit v7.2 software (Ibis Biosciences, USA). The full-length of F gene was used to analyze the evolutionary and phylogenetic tree using MEGA X software (The Biodesign Institute, USA). The Maximum Likelihood method and General Time Reversible model with 1,000 bootstrap replicates were used to construct the phylogenetic tree. Sequences of different genotypes of NDVs from Genbank (National Center for Biotechnology Information, USA) were used for the phylogenetic tree analysis including genotype VIII: QH1 (FJ751918), QH4 (FJ751919); genotype VI: ITA(MN727300), Fontana (AY288992) genotype VII.1: Beh(KX447629), Maz15 (KY205742), Isf16 (KY205741), SMV-2 (KU 201409), SMV-8 (KU 201415), SMV-6(KU 201413), PHL 120807 (MH371031), PHL 206519 (MH371077), PHL 253633 (MH371064), VietNam/480 (GU332647), Go (AF456473), BY7 (AB853930), AGT (AB853929), JS-2 (AF458013), 044(GQ338310), 018(GQ338309), JS-3 (AF450010), CH-A7 (AY028995); genotype VII.2: IBS002(KR074404), IBS005(KR074405), Makassar (HQ697256), Bali(HQ697261), Cilebut(MN727299), VD(MN699676), Sukorejo(HQ697255), Namibia7362(KY747484), Namibia5620(KY747479), Kudus(HQ697260), J11-Jordan(MH614933), AW-d42(MH717067), and AW-by34(MH717064).

2.3 Results and Discussion

After multiple attempts of PCR and sequencing, the complete genome sequences of NDV-GVII-Mega and NDV-GVII-Cimanglid were submitted to Genbank under accession number IDs MN688613 and MN688614 respectively. The table 2.1 below presents these newly emerging NDV strains in this study from the outbreaks as well as their tissue origin, pathotypes, and pathogenicity.

Table 2.1. Isolated Newcastle disease viruses in this study from Indonesian farms

No	Isolate name	Year	Origin	Pathotype	MDT[1] (hrs)	Farm	Sample origin	Vaccination condition
1	Mega	2013	West Java	Velogenic	33	Broiler	Brain	Vaccinated
2	Cimanglid	2015	West Java	Velogenic	60	Layer	Brain	Vaccinated

Sequence analysis revealed that the two strains differ in the amino acid sequence at the C terminus of the F protein cleavage site, which is a key determinant of NDV pathogenicity (de Leeuw et al. 2005a; Panda et al. 2004). The Cimanglid strain encodes the amino acid sequence motif ^{112}RRRKRF^{117}, while the Mega strain encodes ^{112}RRQKRF^{117}. Genomic features of these two new NDV strains are shown in Table 2.2 & 2.3.

Table 2.2 Genomic features of Mega strain

Region	Gene sequence(nt)	3'UTR (nt)	CDS (nt)	5'UTR (nt)	Intergenic region(nt)	Nucleotide length(nt)	Amino acid length(aa)
NP	75-1827	66	141-1610	217	1	1470	406
P	1829-3279	83	1912-3099	180	1	1188	395
M	3281-4521	34	3315-4409	112	1	1095	364
F	4523-6314	46	4569-6230	84	31	1662	553
HN	6346-8347	91	6437-8152	195	47	1716	571
L	8406-15020	-	8406-15020	-		6615	2204

Table 2.3. Genomic features of Cimanglid strain

Region	Gene sequence(nt)	3'UTR (nt)	CDS (nt)	5'UTR (nt)	Intergenic region(nt)	Nucleotide length(nt)	Amino acid length(aa)
NP	182-1934	66	248-1717	217	1	1470	406
P	1936-3386	83	2019-3206	180	1	1188	395
M	3388-4628	34	3422-4516	112	1	1095	364
F	4630-6421	46	4676-6337	84	31	1662	553
HN	6453-8454	91	6544-8259	195	47	1716	571
L	8513-15127	-	8513-15127	-		6615	2204

The phylogenetic tree is generated based on F gene sequences, including the F protein cleavage site, in comparison with 37 type-specific NDV sequences from GenBank. This evolutionary and phylogenetic analysis was also supported by high bootstrap values using MegaX Package (Kumar et al. 2018) suggests these circulating strains belong to genotype VII.2 of NDVs (Fig 2.3), a cause of many recent disease outbreaks in Indonesia. Most of the NDVs isolated in Indonesia before 2009 belonged to genotypes II, III, and VI (Putri et al. 2017) and all recent NDV isolates in Indonesia are genotype VII. The most common sub-

genotypes of NDV in Indonesia are GVII.2 were isolated form deadly outbreaks in vaccinated farms. Nucleotide sequence identity matrix for the coding region of F gene has also shown the VII.2 isolates has 89% identity to the vaccine stains which is the largest distances between circulating GVII isolates and vaccine strains. Interestingly, some recent GVII.2 isolates in Indonesia with very high virulence showed a different motif for cleavage site of the fusion protein as well. These significant differences could contribute to poor protection of NDV vaccines against these circulating strains, which highlights the need for a vaccine development strategy against newly emerged NDV-GVII strains in Southeast Asia.

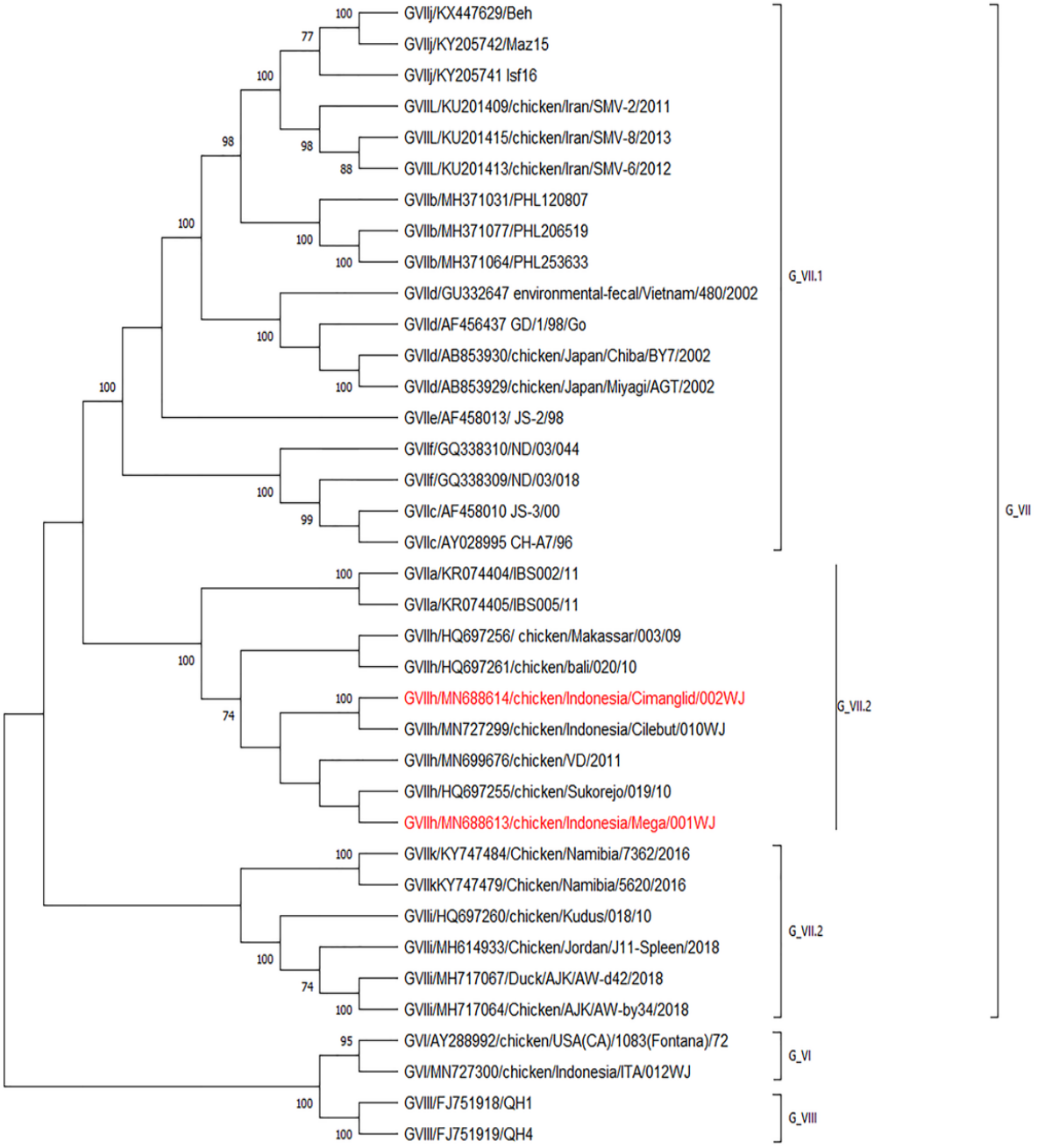

Fig 2.3 Phylogenetic analysis based on the full-length fusion protein gene of representative NDV isolates. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model in Mega X. The tree with the highest log likelihood (-7196.50) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained

automatically by applying Neighbor-Joining 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. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0951)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 33.02% sites). This analysis involved 37 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1647 positions in the final dataset. The vertical lines show genotypes VII with subgenotypes VII.1 and VII.2, genotype VI and VIII. Two virulent strains of subgenotype VII.2 in this study are highlighted in red.

Data availability. The genome sequences for Mega/001WJ and Cimanglid/002WJ were deposited in Genbank with IDs MN688613 and MN688614 respectively. The raw sequence data were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA613298.

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2.4 Original published paper

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GENOME SEQUENCES

Genome Sequences of Newcastle Disease Virus Strains from Two Outbreaks in Indonesia

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ABSTRACT The genomes of two newly emerged Newcastle disease virus strains, chicken/Indonesia/Mega/001WJ/2013 and chicken/Indonesia/Cimanglid/002WJ/2015, from disease outbreaks in chickens in Indonesia are reported. Phylogenetic analysis of different genotypes of Newcastle disease virus using the F gene coding sequences suggests that these two strains belong to genotype VII.2, in class II of avian paramyxoviruses.

Newcastle disease (ND) is one of the most severe infectious diseases of chickens. The causative agent, ND virus (NDV), is a member of the avian genus *Orthoavulavirus* (subfamily *Avulavirinae*, family *Paramyxoviridae*) (1). NDV is a single-stranded, nonsegmented, negative-sense, and enveloped RNA virus with six major structural proteins in the order 3′-NP-P-M-F-HN-L-5′ (2, 3). NDV has been divided into two classes; class I represents avirulent strains, while class II represents virulent and nonvirulent strains (4–6). Recent ND outbreaks have appeared in commercial chickens, even vaccinated flocks, leading to mortality rates of 70 to 80%, and are caused mainly by highly virulent genotype VII NDVs (6, 7).

The two virus strains in this study were collected from two brain samples from vaccinated chickens in two different NDV outbreaks in Indonesia in 2013 and 2015. The samples were processed based on the OIE guidelines for laboratory procedures for isolating the virus (8) and then were inoculated into embryonated chicken eggs, followed by collection of allantoic fluid. RNA purification was performed using the viral RNA minikit (Qiagen, USA). cDNA libraries were prepared using random hexamers with the stranded mRNA-Seq kit (Kapa Biosystems, USA) according to the manufacturer's instructions. The resulting cDNAs were sequenced using the Illumina MiSeq platform 600-cycle kit v3, generating 2 × 300-nucleotide reads, and the library size was checked on a Bioanalyzer 2100 using the high-sensitivity DNA kit (Agilent Technologies, Germany). Adaptors were removed using Trimmomatic v0.36 (9); 616,471 and 794,856 reads for samples 1 and 2, respectively, were *de novo* assembled using Unicycler v0.4.4 with default parameters (10) and visualized with Bandage (11). Assembled contigs from each sample were compared to the NCBI nonredundant/nucleotide collection using BLASTn (12). Two NDV contigs with a genome GC content of 46% and a length of 15,192 nucleotides were identified for the chicken/Indonesia/Mega/001WJ/2013 (Mega/001WJ) and chicken/Indonesia/Cimanglid/002WJ/2015 (Cimanglid/002WJ) strains. The Mega/001WJ and Cimanglid/002WJ contigs had average coverage of 573× and 1,837×, respectively, and 97.97% and 98% identity to the Sukorejo strain (GenBank

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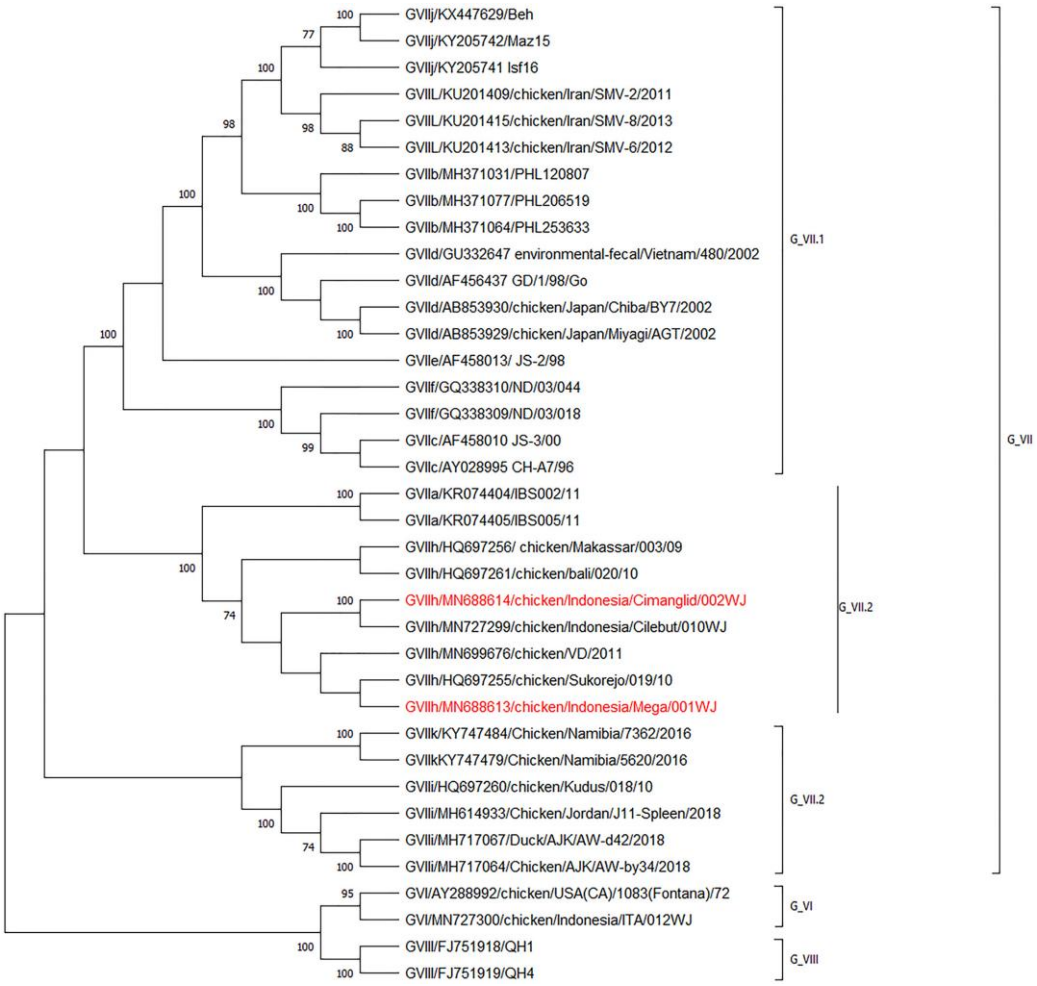

FIG 1 Phylogenetic analysis based on the full-length fusion protein gene of representative NDV isolates. The evolutionary history was inferred by using the maximum likelihood method and general time-reversible model in MEGA X. The tree with the highest log likelihood (−7,196.50) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with a superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories [+G; parameter, 1.0951]). The rate variation model allowed for some sites to be evolutionarily invariable (+I; 33.02% of sites). This analysis involved 37 nucleotide sequences. Codon positions included were first, second, third, and noncoding. All positions with <95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1,647 positions in the final data set. The vertical lines show genotype VII, with subgenotypes VII.1 and VII.2, genotype VI, and genotype VIII. The two virulent strains of subgenotype VII.2 in this study are highlighted in red.

accession number HQ697255), respectively. A gap in the sequence of Mega/001WJ was closed using reverse transcriptase PCR (Qiagen) and Sanger sequencing (13), and BioEdit v7.2 was used to merge sequences (14). Geneious release 10.1.3 and ORFfinder were used to annotate genes and to confirm open reading frames, respectively (12).

Sequence analysis revealed that the two strains differed in the amino acid sequence at the C terminus of the F protein cleavage site, which is a key determinant of NDV

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pathogenicity (15, 16). The Cimanglid/002WJ strain encodes the amino acid sequence motif ^{112}RRRKRF^{117}, while the Mega/001WJ strain encodes ^{112}RRQKRF^{117}. Phylogenetic analysis carried out on F gene sequences using MEGA X (17) suggests that these circulating strains belong to NDV genotype VII.2 (Fig. 1), a cause of many recent disease outbreaks in Indonesia. The amino acid identities of NP, P, M, F, HN, and L proteins between the current virus strains and the LaSota vaccine strain that is most commonly used in Indonesia are 92%, 81%, 88%, 89%, 85%, and 94%, respectively. These differences could contribute to poor protection against these strains by NDV vaccines, which highlights the need for a vaccine development strategy against newly emerged NDV strains in Southeast Asia.

Data availability. The genome sequences for Mega/001WJ and Cimanglid/002WJ were deposited in GenBank with accession numbers MN688613 and MN688614, respectively. The raw sequence data were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA613298.

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CHAPTER 3: TRANSCRIPTOME ANALYSIS OF CHICKEN SPLEENS REVEALS A KEY TO THE MOLECULAR PATHOGENESIS OF VIRULENT NEWCASTLE DISEASE VIRUS GENOTYPE VII (NDV-GVII)

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Transcriptome analysis of chicken spleens reveals a key to the molecular pathogenesis of virulent Newcastle disease virus genotype VII (NDV-GVII)

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Abstract: Genotype VII strains of the Newcastle Disease Virus **(**NDV-GVII) cause lymphocyte depletion in infected birds and are responsible for devastating economic losses in the poultry industry. However, the underlying molecular pathogenesis of this immunopathological tropism is not fully understood. In this study, specific pathogen-free (SPF) chickens were experimentally infected with NDV-GVII originating from recent outbreaks and the transcriptome of their spleens was characterized. Virulent NDV-GVII significantly downregulates immunologically immune regulating pathways including fMLP signalling in neutrophils, *PI*3K signalling in B Lymphocytes, Fcy receptor-mediated phagocytosis in macrophages and monocytes, leukocyte extravasation signalling and NF-kB activation by viruses, which lead to widespread immunosuppression and enhanced replication of the virus. As a result, the host's immune response is diminished, delayed, incomplete or displays overly strong induction after delay that can cause severe tissue damage. Moreover, PPI network analysis revealed that the top three gene modules are significantly enriched in phagocytosis, immune response-related terms, and glutamate receptor signalling. We

identified novel genes EGF, LPAR5, AGT, AGTR1, RAC2, CD4, CD3D, IL7R, NPY, GRM3, and GRAP2 as potential biomarkers. We have identified novel genes and signalling pathways regulating the host immune response to the virus, and shown how targeting of the virus to lymphocytes, causing their destruction, is the likely mechanism by which highly pathogenic NDV-GVII negates the host immune system.

Keywords: Newcastle disease virus; NDV-GVII; RNA-Seq; spleen; chicken; lymphocyte depletion; immunosuppression

3.1 Introduction

NDV-GVII viruses are globally predominant virulent strains, especially in Southeast Asia, and have been responsible for the fourth and fifth NDV pandemics worldwide (Miller et al. 2015). NDV-GVII strains are predicted to be highly pathogenic and can cause mortality in up to 100% of chickens in challenge experiments, and up to 80% on vaccinated poultry farms (Dimitrov, Kiril M, Ramey, Andrew M, et al. 2016; Ebrahimi et al. 2012; Miller et al. 2015). Additionally, the most virulent NDV-GVII strains trigger severe lymphocyte depletion and extensive necrosis, while other virulent strains cause milder lesions (Hu, Z. et al. 2015). Previous studies indicated that virulent NDV infections induced strong innate immune and intensive inflammatory responses, resulting in tissue damage and cell death in lymphoid organs (Hu, Z et al. 2012; Hu, Zenglei et al. 2015; Liu, W-Q et al. 2012; Rue et al. 2011; Zhang, T et al. 2019). Understanding the molecular pathogenesis of NDV-GVII is crucial for preventing or controlling this devastating disease. Although there have been several efforts to characterize pathogenesis of different NDV genotypes and pathotypes (Baier, Pfeifhofer & Thuille 2012; Hu, Z. et al. 2015; Kristeen-Teo et al. 2017; Rasoli et al. 2014), this appears to be the first study of changes in the host transcriptome after infection with NDV-GVII and to correlate them with clinical and immunopathological outcomes.

RNA sequencing (RNA-Seq) is now being applied to the molecular pathogenesis of viruses, providing deeper insights into which genes are differentially expressed and likely to cause disease upon infection (Radford et al. 2012). RNA-seq has also been used to identify differential gene expression in various host tissues, including spleen, trachea, harderian gland and lung, in response to infection with the La Sota strain of NDV (Deist, Melissa S et al. 2017; Deist et al. 2018; Zhang, Jibin et al. 2018). Similarly, avian interferon-stimulated genes and virus–induced cellular responses in chicken embryo fibroblasts (CEFs) infected in vitro with NDV strain Herts/33 have been identified using RNA-seq (Liu, W et al. 2018).

Transcriptome analysis of the spleen of NDV-GVII infected chickens has been discussed, mainly focusing on the apoptosis and necrosis pathways that contribute to the disease during infection (Rabiei et al. 2021). However, the general dynamics of chicken spleen transcription following infection with virulent NDV-GVII in vivo, are not fully understood. The spleen is a major immune organ where B and T cells mature in adult birds, and is known to be infected early after NDV exposure(Brown, C, King & Seal 1999; Cesta 2006). Hence, characterizing the host transcriptome response using RNA-seq may provide a more comprehensive understanding of host-virus interplay and shed light on the molecular mechanism of the lymphotropic pathotype of the NDV-GVII. In this study, pathway and protein-protein network analyses were carried out to identify possible hub genes and novel pathway clusters, to help provide a better insight into their roles in the molecular mechanisms of disease pathogenesis.

3.2 Materials and Methods

3.2.1 Virus

NDV-GVII isolate, Chicken/Indonesia/Mega/001WJ/2013 (Accession number: MN688613.1) was isolated from the brain of a vaccinated broiler chicken during an NDV outbreak in West Java, Indonesia in 2013 (Doan et al. 2020). This virus is referred to as Mega throughout this chapter. The plaque purified isolate was then propagated in 9-day-old specific pathogen-free (SPE) embryonated chicken eggs according to the standard OIE method (OIE 2018). This NDV isolate's pathogenicity was assessed using the Mean Death Time (MDT) and Intra-Cerebral Pathogenicity assays. To determine the MDT for this isolate, a ten-fold serial dilution of infected allantoic fluid from the isolate (10-3 to 10-9) was made in sterile PBS. A 100µl aliquot of each dilution was inoculated into the allantoic cavity of five 9-day-old embryonated SPF chicken eggs and incubated at 37°C for seven days. The eggs were examined twice a day, and the time of embryo death was recorded. The ICPI has been measured at the time of isolation of the Mega strain. Based on recorded data the ICPI was 1.58 (out of a maximum value of 2 for ITA (GVI) strain, accession number: MN727300). The pathotype of the Chicken/Indonesia/Mega/001WJ/2013 isolate in this study was confirmed to be virulent, having an MDT of 33 hr in embryonated chicken eggs (< 60 hr being considered velogenic) and ICPI of 1.58 (OIE 2018).

3.2.2 Animal experiment and sample collection

Animal ethics: The ethics committee approved all experimental procedures at the Indonesian Research Centre for Veterinary Science (Bbalitvet), Bogor, Indonesia, reference number A.H./2015/003. A specialized veterinarian managed the experimental chickens based on the National Health and Medical Research Council of Australia guidelines (Council 2013). The birds were monitored daily for clinical signs, morbidity, and mortality. All birds were bled via a brachial vein or by cardiac puncture as a terminal step just after euthanasia. Twenty-one-day-

old specific pathogen-free (SPF) layer chicks were divided into two groups of 10 chicken and reared in separate isolators at biosafety level 3 (BLS3) biocontainment at the Indonesian Research Center for Veterinary Science (Bbalitvet). The first group was a negative control and received no virus, while the second group was challenged with Mega. A serum sample from each chicken was subjected to a hemagglutination inhibition test to ensure chickens were seronegative for NDV at the beginning of the study. At 35 days of age, the experimental birds were inoculated by intraocular and intranasal instillation with 100µL of 10^2 EID50 of live virus (Alexander, D. J., Manvell & Parsons 2006; Miller et al. 2013). PBS was used as the placebo for non-infected birds. On day 3, after the challenge and due to the severe illness and death amongst chickens, all surviving birds were euthanized and necropsied to collect tissue samples for RNA isolation. Spleens, cloacal and tracheal swabs were freshly collected and immediately placed into RNA-later and stored at -80°C.

3.2.3 Total RNA isolation

Total RNA was extracted from 100mg of spleen from each bird, using a commercial mirVana™ miRNA Isolation Kit (Ambion, Thermo Fisher, USA) according to the manufacturer's instruction. RNA quality was measured using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA purity and integrity were also measured using a 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Samples with a high RNA Integrity Number (RIN ≥ 8) were selected for cDNA library construction and RNA-seq analysis.

Samples of cloacal, lung, brain, spleen, bursa, liver and tracheal samples from each chicken were taken for virus isolation and qRT-PCR. The viral RNA was extracted from samples using QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) and quantified as described above. Five µl of extracted RNA was converted to cDNA using a SuperScript™ IV First-Strand Synthesis System (Invitrogen, CA, USA) as per manufacturer's instruction.

Absolute quantification for detection of viral load by qRT-PCR was performed using primers for the F gene, (FF = AAAGTGGTGACACAGGTCGG, and FR = CCGATGTATTGCCGCTCAAG), corresponding to nucleotides 5488 to 5507 and 5632 to 5613 and generating a 145 bp amplicon. Quantitative polymerase chain reaction (q-PCR) was carried out using QuantiFast SYBR® Green PCR Kit (QIAGEN, Hilden, Germany). The reaction was analysed with an Illumina, Eco Real-Time PCR machine (Illumina Inc, California U.S.A.) with initial denaturation at 95°C for 3min followed by 40 cycles of 95°C for 10s and 60°C for 30s. Each qRT-PCR reaction was repeated three times in triplicate.

3.2.4 cDNA library construction and RNA sequence

For each sample, an Illumina TrueSeq RNA sample preparation kit (Illumina. California U.S.A.) was used to construct a library from 0.5µg of total RNA from each sample, following the manufacturer's protocol. The poly-A mRNA was purified using oligo(dT) magnetic beads and fragmented via divalent cations and heat from each replicate. The first and second strand cDNA were synthesized and subjected to end repair. A-tailing and sequence adapters were ligated before DNA fragments were enriched via PCR amplification, following by purification using 2% agarose gel electrophoresis. Paired-end sequencing was performed on an Illumina NovaSeq S1 300 cycle instrument (Illumina. Inc, San Diego, CA, USA) with the read length of 2x150 bp.

3.2.5 Data analysis

Raw RNA-seq reads from all samples were initially checked for quality using FASTQC v0.11.4 (Andrews 2010) and then trimmed using TrimGalore v0.4.2 (Krueger & Galore 2015). The reads with a minimum length of 100 bp and where all the bases had a minimum Phred score of 10 were selected. AdapterRemoval v2.2.1 was used to eliminate adapter sequences and the reads were checked again with FASTQC (Schubert, Lindgreen & Orlando 2016). Reads were then mapped to the chicken reference genome (GRCg6a) using HISAT2

v2.1.0 (Kim, D, Langmead & Salzberg 2015) and sorted with SAMtools v1.8 (Li, H et al. 2009). Reads were counted for each gene using featureCounts v1.6.3(Liao, Smyth & Shi 2014) with genes defined using Ensembl annotation v97.

3.2.6 Differential gene expression and pathway analysis

To identify changes in gene expression, a limma-voom pipeline (Liu, R et al. 2015; Ritchie et al. 2015) was used to compare reads per gene grouped by infected and control. Out of 24362 expressed genes, 9698 were removed as lowly expressed, based on a count per million (CPM) of less than 1 in two samples or more. The counts were normalised to correct for differences in library size using the trimmed mean of M values (TMM) method (Robinson, Mark D & Oshlack 2010). Samples and individual observational level of each expressed gene was weighted using Voom (Law, CW et al. 2014) to account for heterogeneity in their expression level. Moderated t-statistic tests were used to establish differential expression levels between samples. The R statistical package software EdgeR (Robinson, M. D., McCarthy & Smyth 2010) was used to perform differential expression analysis. Differentially expressed genes (DEGs) between control and infected groups were selected using a false discovery rate (FDR) < 0.05.

Genes with FDR < 0.05 and absolute $\log_2$ Fold Change ($\log_2$FC) ≥ 1 were consider to be differentially expressed (DEGs), and were analysed using the Ingenuity Pathway Analysis software (IPA, QIAGEN, Redwood City, CA) to identify the pathways and biological functions regulating the host immune responses (Krämer et al. 2014).

3.2.7 Protein-protein interaction (PPI) network construction and hub gene identification

A PPI network of DEGs was constructed using the publicly available Search Tool for Retrieval of Interacting Genes (STRING) database (Szklarczyk et al. 2019), a biological database and source of protein-protein interaction predictions. PPIs with a confidence score < 0.4 and disconnected nodes were removed. Results were visualized using Cytoscape software

3.8.0 (Shannon et al. 2003). The CytoHubba v0.1 plugin (Chin et al. 2014) of the Cytoscape platform was used to identify the hub genes in the PPI network based on the hub score calculation using 5 topological analysis methods utilized by this application: Maximal Clique Centrality (MCC), Maximum Neighbourhood Component (MNC), Edge Percolated Component (EPC), Degree and Closeness. An online tool, http://bioinformatics.psb.ugent.be/webtools/Venn/, was employed to generate a Venn plot for the intersecting genes encoding the core proteins that may represent critical genes having crucial functions in biological pathway regulation. Additionally, MCODE v1.5.1 (Molecular Complex Detection) (Bader & Hogue 2003), an application in Cytoscape to find densely interconnected regions in large protein-protein interaction networks, was used to construct the PPI network modules with parameter settings: degree cutoff = 2, node score cutoff = 0.2, k-core = 2, maximum depth = 100. The highest scoring network is considered as the most significant.

Functional annotation analyses for the top three modules were performed using ClueGO v2.5.6 (Bindea et al. 2009) and CluePedia plug-ins (Bindea, Galon & Mlecnik 2013) in Cytoscape (Cline et al. 2007). The right-sided hypergeometric test with Benjamini-Hochberg correction was used to test for significant enrichment (P.value < 0.05) of pathways, and a corrected kappa score of 0.4 was used to calculate the association strength between biological pathways.

3.2.8 Validation of RNA-seq data by quantitative PCR (qRT-PCR)

Total RNA extracted from spleen after NDV infection was used to measure changes in the transcripts of eight candidate genes by qPCR. Candidate genes were selected based on their role in molecular pathways of disease in similar chicken viral diseases. Gene-specific primers were designed using The National Center for Biotechnology Information (NCBI) software for exon-exon spanning or intron inclusion. The primer sequences were then

checked for secondary structure and other characteristics using OligoAnalyzer tool (https://sg.idtdna.com/pages/tools/oligoanalyzer) and synthesized by Sigma Aldrich (NSW, Australia) (Table 3.1). qPCR of 10-fold serial dilutions followed by gel electrophoresis with 2% agarose were also used to optimize the PCR amplification efficiency and candidate gene specificity. The geometric means of Ct values of two control genes, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (beta-actin), were used for normalization. RNA samples from spleen tissues were used to synthesize cDNA for quantitative PCR using QuantiTect Reverse Transcription kit (QIAGEN *GmbH*, Hilden, *Germany*) according to the manufacturer's instructions. Genomic DNA was removed by incubating in gDNA Wipeout buffer at 42°C for 2 minutes. A PCR master mix of the total volume of 20 µl was prepared as per the manufacturer's protocol. The cDNA and gene-specific primers were added to the PCR master mix. All samples, along with negative and positive controls were dispensed to 384 well plates using an automated Corbett robot (Corbett Research, Sydney, Australia). The thermal cycling profile consisted of initial heat denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and combined annealing/extension at 60°C for 30 seconds. To assess the specificity of amplification, a ramp of 60-99°C was added to the melting curve step. All the reactions were performed in triplicate. Negative controls lacked template. Expression differences between challenged and unchallenged birds were measured using the $2^{-\Delta\Delta Ct}$ method, and the expression level of target genes was normalized against the control genes GAPDH and ACTB (Khan, Roberts & Wu 2017). The relationship between RT-qPCR and RNA-seq data were calculated using linear regression (Pearson correlation).

474 Table 3.1. Primer sequences for qRT-PCR validation of RNA-seq data

gene symbol	Primer Sequence	fragment size (bp)	Ta °C	PCR Efficiency (%)	Accession No. /References
OASL	Forward: GGAGTCAGCATCACCAGTCC Reverse:CTGAATCACCTGCCCCAGTG	144	64	109	XM_015293006.2
IFIT5	Forward:AACTGGACAAGGCACAAGAGG Reverse: CTCGGTAGCAGAGTCCTAGC	93	56	102	NM_001320422.1
MX1	Forward: CTTCACGTCAATGTCCCAGC Reverse: GGCGTTTATTTGCTCCAGTGA	78	58	100	GU256272.1
STAT1	Forward: CTGTTTGTTCCAGCGGGATGAC Reverse: CGTTGTTGGCTGCGTGTTCC	165	57	94	NM_001012914.1
RPS27A	Forward: TCTTCACCTTGTGCTGAGACTG Reverse:GCCGTTCTCATCCACCTTGTAGT	139	60	102	NM_001287205.1
CXCR4	Forward: GCATGGACGGTTTGGATCTGT Reverse: CCAATCTCCTCCGAGCCATT	70	60	100	NM_204617.2
IL16	Forward: GCTTCAGTCTGGAAGGTGG Reverse: TGTTCCAACGAGGTCCCTTT	88	58	97	NM_001277996.1
RAC2	Forward: AGGATTACGACAGGCTGAGGC Reverse: GATGCTGGGCTGACAAGGGA	82	61	110	NC_006088.5
GAPDH	Forward: GAAGGCTGGGGCTCATCTG Reverse: CAGTTGGTGGTGCACGATG	150	60	107	
ACTB	Forward: CCAGACATCAGGGTGTGATGG Reverse: CTCCATATCATCCCAGTTGGTGA	137	60	98	

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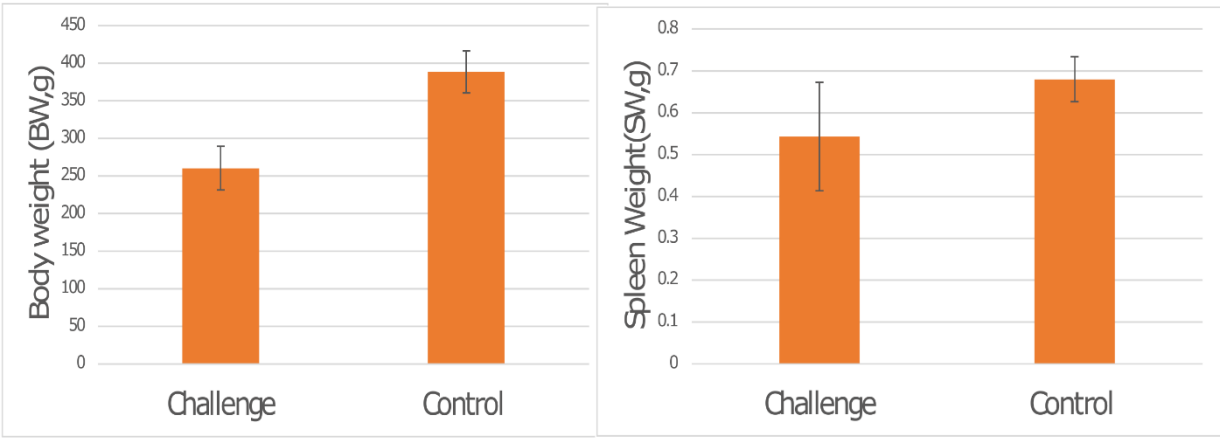
3.3 Results

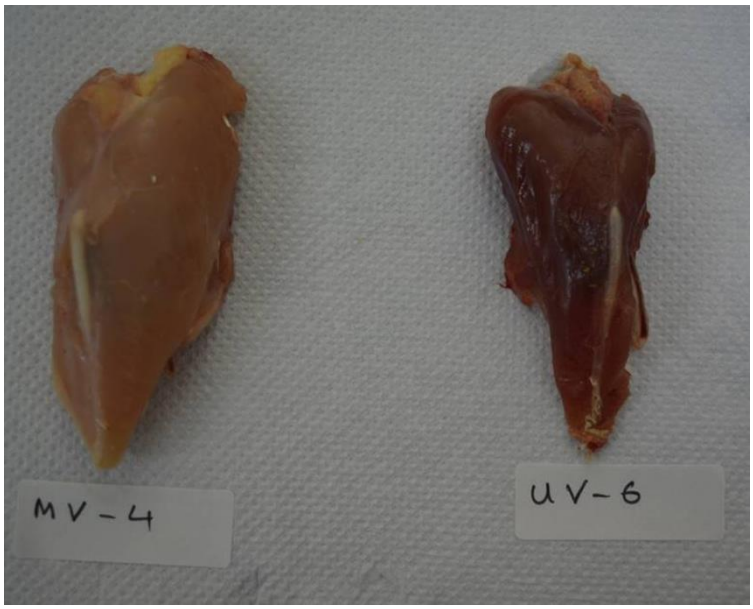
3.3.1 Clinical evaluation of challenge and control groups

Within first 48 hours of the challenge experiment, five of 10 experimentally infected chickens died and the other five were very sick. On the first day of the experiment the sick birds stopped drinking water and consuming food. The sick birds were also reluctant to move and showed hyperaemia or spot haemorrhages on their left leg with minor lacrimal discharges. Post-mortem examinations were conducted for all dead and euthanized chickens according to the Bermudez and Stewart-Brown protocol (Saif 2008). Results indicated that the most common changes in challenged birds were significant hyperemia and decreased size of the spleen. The body and spleen weights of diseased birds at the early stage of NDV-GVII infection were significantly lower than those of the control group ($P < 0.05$; Fig 3.1). The other notable and unique post-mortem finding was dark breast muscles in all challenged birds (Fig 3.2).

Furthermore, hemorrhages in proventriculus (8 out of the 10 cases), conjunctival hemorrhages (10 out of the 10 cases) and hyperemia and hemorrhages in the left leg (8 out of the 10 cases) were also observed. The leg hemorrhages have not been reported in any other NDV experiment. Additionally, the qPCR results for the F gene from different tissues and swabs indicated that the *Ct* values of lymphoid tissue samples (spleen and bursa) were considerably lower ($P<0.001$), meaning that the viral load was significantly higher in comparison with other organs (Fig 3.3).



499 Fig 3.1. Body and spleen weight between challenge and control groups(n=10).



500 Fig 3.2. Muscular darkness in the challenged bird (right) compared to the control bird (left)

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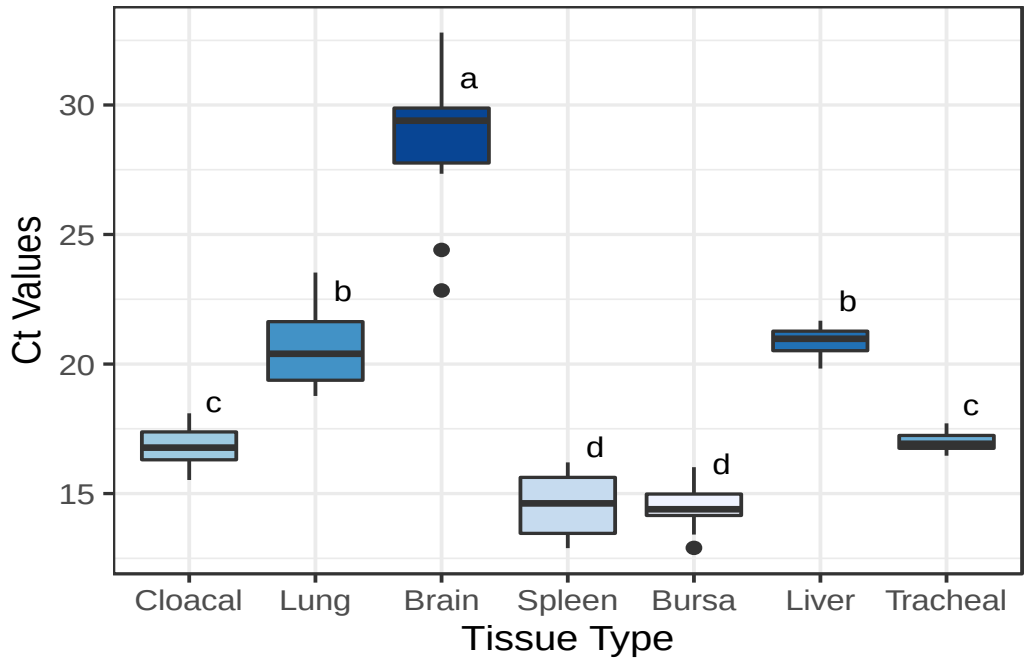

Fig 3.3. Quantitative PCR results based on the *Ct* values for different tissues and swabs from challenged birds (n=10). The letters show significant difference at P<0.001 by the Tukey test. A lighter colour in the boxplot indicates a lower median of Ct values.

3.3.2 Summary statistics of the RNA sequencing reads

RNA from six spleens was sequenced. A total of 562,004,121 2 × 150bp paired-end (PE) raw reads were generated, ranging from 29 to 145M reads per sample. After cleaning, 26 to 137M reads remained, with approximately 90% of reads per sample mapping to the chicken reference genome (GRCg6a). The majority mapped uniquely to the reference genome, accounting for at least 75% of the clean reads, whereas about 2% mapped to multiple locations in the genome. The feature summary of reading counts is shown in Table 3.2

Table 3.2. Descriptive statistics of read count and mapping to the reference genome

Group	Raw reads	Clean reads	Hisat2 mapping (%)	Uniquely mapped reads		Multi mapped reads		Unmapped reads	
				Counts	%	Counts	%	Counts	%
Treatment_1	145,682,503	137,041,556	88.67	106,270,232	77.55	2,339,244	1.71	28,432,080	20.75
Treatment_2	140,667,468	125,491,032	90.33	94,866,709	75.6	5,294,448	4.22	25,329,875	20.18
Treatment_3	91,532,141	87,257,038	90.42	72,794,736	83.43	1,484,952	1.7	12,977,350	14.87
Control_1	29,105,264	26,879,254	88.34	21,207,283	78.9	331,055	1.23	5,340,916	19.87
Control_2	113,657,819	102,102,063	87.89	79,235,036	77.6	1,468,618	1.44	21,398,409	20.96
Control_3	41,358,926	38,723,858	88.54	30,377,276	78.45	503,808	1.3	7,842,774	20.25

3.3.3 Differential expression in response to genotype VII virulent NDV infection

mRNA gene expression in the spleens of infected versus uninfected NDV chickens was analysed. 6,361 of 14,664 genes were found to be differentially expressed, with 2,855 down-regulated and 3,506 up-regulated. Of these DEGs, 1,218 with an absolute $\log_2$ fold change > 3 were selected for further analysis. The five most up-regulated genes were AGT (Angiotensinogen), SLC1A3 (Solute Carrier Family 1 Member 3), PLP1 (Proteolipid Protein 1), GFAP (Glial Fibrillary Acidic Protein), and GPM6A (Glycoprotein M6A). CD5 (CD5 Molecule), KCNH4 (Potassium Voltage-Gated Channel Subfamily H Member 4) AICDA (Activation Induced Cytidine Deaminase), FLT3 (Fms Related Receptor Tyrosine Kinase 3), and ANKDD2 (Ankyrin Repeat Domain 2) were the five most down-regulated genes. A volcano plot of the DEGs is shown in Fig 3.4a, and a hierarchical clustering analysis of the top 20 DEGs showing a clear difference in the pattern of DEGs between treatment and control groups is depicted in Fig 3.4b.

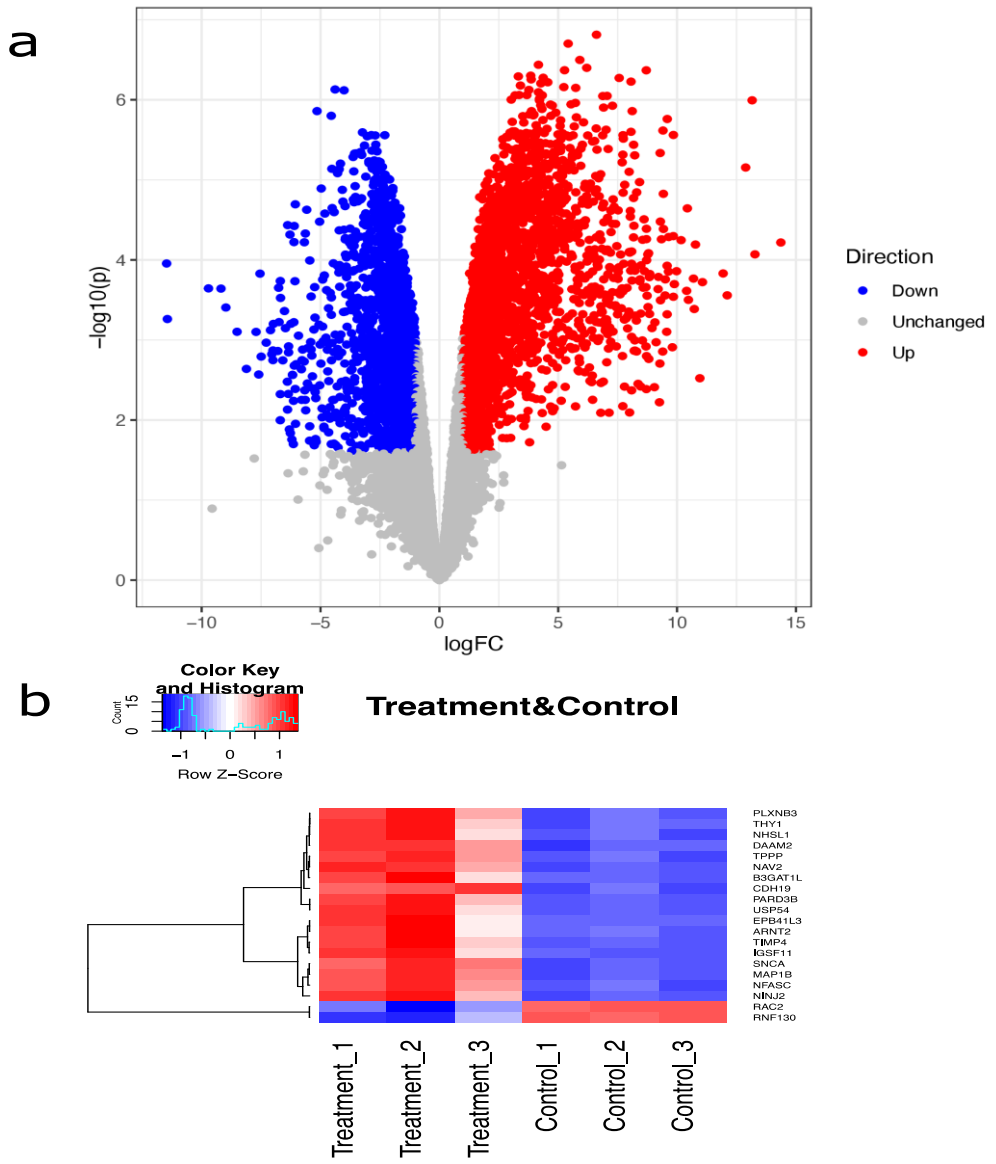

Fig 3.4 A volcano plot and Hierarchical clustering plot between treatments and controls. a. A volcano plot shows differentially expressed genes. The red and blue dots represent upregulated and downregulated genes, respectively. Grey dots represent genes that are under the cut-off point and considered as not differentially expressed. The x-axis shows the log2FC, and the y-axis illustrates the –log10 value (P.value). b. Hierarchical clustering analysis of the top 20 significantly DEGs between treatment and control groups based on FDR < 0.05 and $|\log2FC| \geq 1$.

3.3.4 Biologically functional enrichment analysis of differentially expressed genes

To better understand the potential biological functions and disease pathways regulating the host immune response, all DEGs with an absolute $\log_2$ fold change > 1) were tested for significantly enriched functions. The majority of DEGs were enriched in: cellular immune response; cellular growth, proliferation and development; cytokine signalling; cellular stress and injury; and humoral immune response ($p<0.05$, z-score < -2) (Fig 3.5). Almost all these enriched pathways were significantly downregulated, whereas the top five inhibited pathways were fMLP signalling in Neutrophils, PI3K signalling in B Lymphocytes, Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes, Leukocyte Extravasation signalling and NF-kB activation by viruses. The DEGs including PI3K (phosphoinositide 3 –kinase) family genes such as PI3Kp85, PI3KCA, PI3KC2G, PI3KC3, PI3KR1/5/6; IkB kinase (IKK) complex (IKBKE, IKBKB); NFAT (Nuclear factor of activated T cells) such as NFAT5/C2/C3 and NF-kB (Nuclear factor-kB) families were involved in these multiple pathways. In addition to canonical pathways, DEGs were also grouped by diseases and functions. Consistent with the results of canonical pathway analysis, a number of categories of annotation functions and diseases showed significant decline following viral infection including quantity of leukocytes, lymphatic system cells, lymphoid cells, lymphoid tissue – all associated with the destruction of lymphoid tissues (Table 3.3).

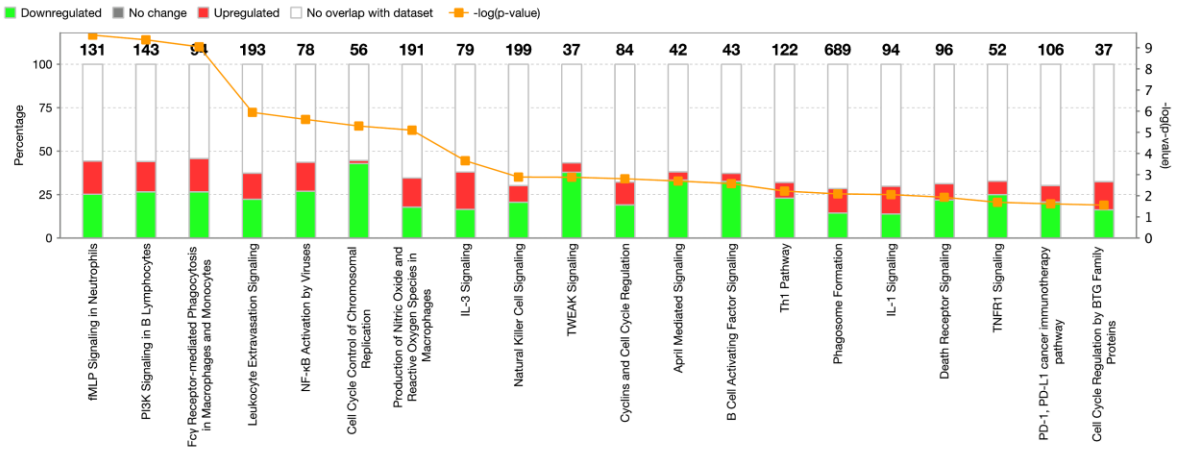

Fig 3.5. The canonical pathways are predicted by IPA to be activated or inhibited for DEGs between challenged and control groups. The top significant pathways were filtered by a -log(p-value) of ≥ 2 and |Z-score| of ≥ 2. The yellow line represents the -log(p-value) of each pathway. The stacked bars indicate the percentage of genes distributed according to regulation that were significantly downregulated (green) and upregulated (red) in each canonical pathway. The genes in a given pathway that were not found in our DEGs list is termed No change (grey) or No overlap with dataset (white). The numerical value at the top of each canonical pathway represents the total number of genes in the whole canonical pathway.

582 Table 3.3. The top 20 diseases or functions annotation by p-value

Diseases or Functions Annotation	p-value	Predicted Activation State	Activation z-score
Organismal death	2.67E-41	Decreased	-2.371
Morbidity or mortality	1.11E-39	Decreased	-2.261
Cell cycle progression	2.79E-17	Decreased	-2.283
Quantity of leukocytes	8.89E-17	Decreased	-2.513
Quantity of lymphatic system cells	1.26E-16	Decreased	-2.933
Quantity of blood cells	3.76E-16	Decreased	-2.857
Quantity of lymphoid cells	9.70E-16	Decreased	-3.261
Quantity of lymphocytes	9.91E-16	Decreased	-3.23
Quantity of mononuclear leukocytes	1.76E-15	Decreased	-3.07
Quantity of lymphoid tissue	7.84E-14	Decreased	-2.361
Organ Degeneration	2.55E-13	Decreased	-2.504
The proliferation of lymphatic system cells	2.63E-13	Decreased	-3.298
The proliferation of immune cells	2.44E-12	Decreased	-2.821
Homeostasis of blood cells	2.99E-12	Decreased	-4.536
Proliferation of lymphocytes	3.51E-12	Decreased	-3.204
The proliferation of mononuclear leukocytes	5.09E-12	Decreased	-3.019
Lymphocyte homeostasis	7.18E-12	Decreased	-4.555
Lymphopoiesis	7.24E-12	Decreased	-5.153
Homeostasis of leukocytes	8.58E-12	Decreased	-4.559
Differentiation of mononuclear leukocytes	1.28E-11	Decreased	-5.206

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3.3.5 PPI network analysis and hub gene identification

The STRING database was used to construct a Protein-Protein Interaction (PPI) network for the selected DEGs. A PPI network consisting of 733 nodes and 1,692 edges with a high confidence interaction score of 0.7 was visualized (Fig 3.6). Essential genes were identified using five centrality methods simultaneously, as described in Methods (Fig 3.7 &Table 3.4). The top 50 DEGs of identified in this way were then used to identify hub genes. The 11 hub genes (EGF, LPAR5, AGT, AGTR1, RAC2, CD4, CD3D, IL7R, NPY, GRM3, and GRAP2) may be genes critical in the molecular pathogenesis of disease production. To identify the functional and disease potential of DEGs, the MCODE plug-in of Cytoscape was used to construct the candidate modules from the DEG PPI network, using a degree cut-off of 2, node score cut-off of 0.2, k-core of 2, and maximum depth of 2. The top 3 most significant modules, with scores of 21.051; 10 and 7.23, respectively, were identified (Fig 3.8). Eleven key genes were found in the first module, showing the importance of this module in causing disease. Gene ontology (GO) functional annotation of gene modules was also carried out. The genes in the first and third gene modules were mainly associated with phagocytosis and immune response-related terms. Specifically, DGEs in the first gene module were significantly enriched in the immune response-activating cell surface receptor signaling pathway, accounting for 32.53% of terms per group, followed by endocytosis with 22.76% and leukocyte migration pathway with 16,26%. GO term analysis of the second gene module showed DEGs significantly ($p < 0.001$) enriched for neuropathological–regulating biological functions including glutamate receptor signaling pathway, long-term synaptic potentiation, and glial cell development pathways. The third gene module, containing immune-stimulated genes such as IFIT5, STAT1, OASL, RSAD2, MX1, TLR3, CMPK2, and ISG12, was highly enriched for defense response to virus and negative regulation of viral replication. PPI analysis results match well with other pathway predictions.

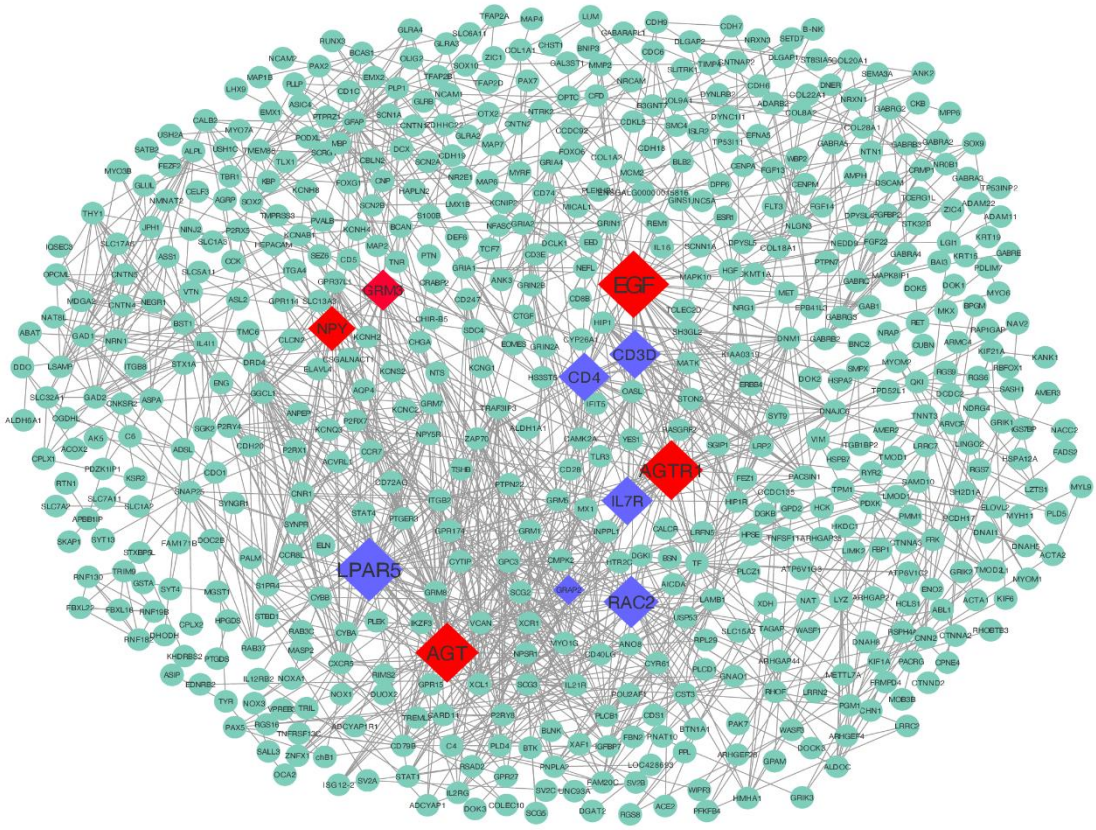

Fig 3.6. The PPI network of DEGs, a total of 733 nodes and 1,692 edges, were identified. The diamond red and yellow nodes indicate upregulated and downregulated hub genes, respectively. The hub node size indicates the connectivity degrees.

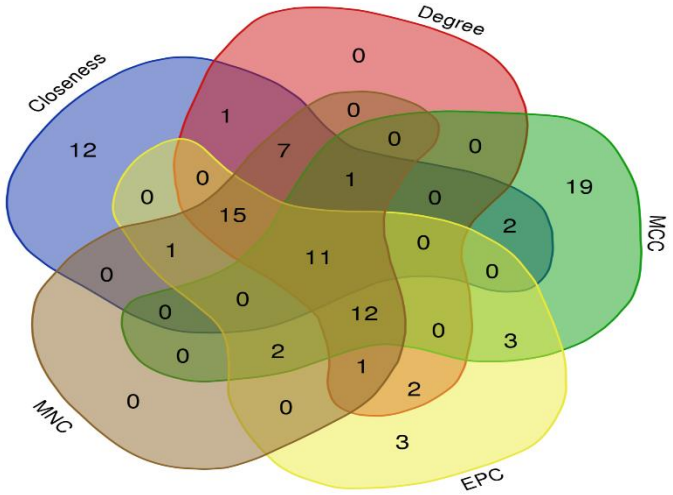

Fig 3.7. Venn diagram for hub genes generated by five algorithms, including Degree, MCC, EPC, MNC, and Closeness. The different colours indicate the distinct methods. The commonly accumulated DEGs are found in the overlaps.

Table 3.4. The 11 hub genes of Newcastle disease caused by virulent genotype VII NDV.

Symbol	Name	Degree	logFC	FDR
EGF	Epidermal Growth Factor	36	8.06	0.0004
LPAR5	Lysophosphatidic Acid Receptor 5	33	-3.86	0.0133
AGT	Angiotensinogen	31	14.37	0.0009
AGTR1	Angiotensin II Receptor Type 1	30	4.97	0.0014
RAC2	Rac Family Small GTPase 2	28	-4.39	0.0004
CD4	CD4 Molecule	25	-4.91	0.0037
CD3D	CD3d Molecule	25	-6.06	0.0006
IL7R	Interleukin 7 Receptor	25	-3.41	0.0254
NPY	Neuropeptide Y	24	5.86	0.0033
GRM3	Glutamate Metabotropic Receptor 3	22	3.58	0.0055
GRAP2	GRB2 Related Adaptor Protein 2	13	-5.23	0.0031

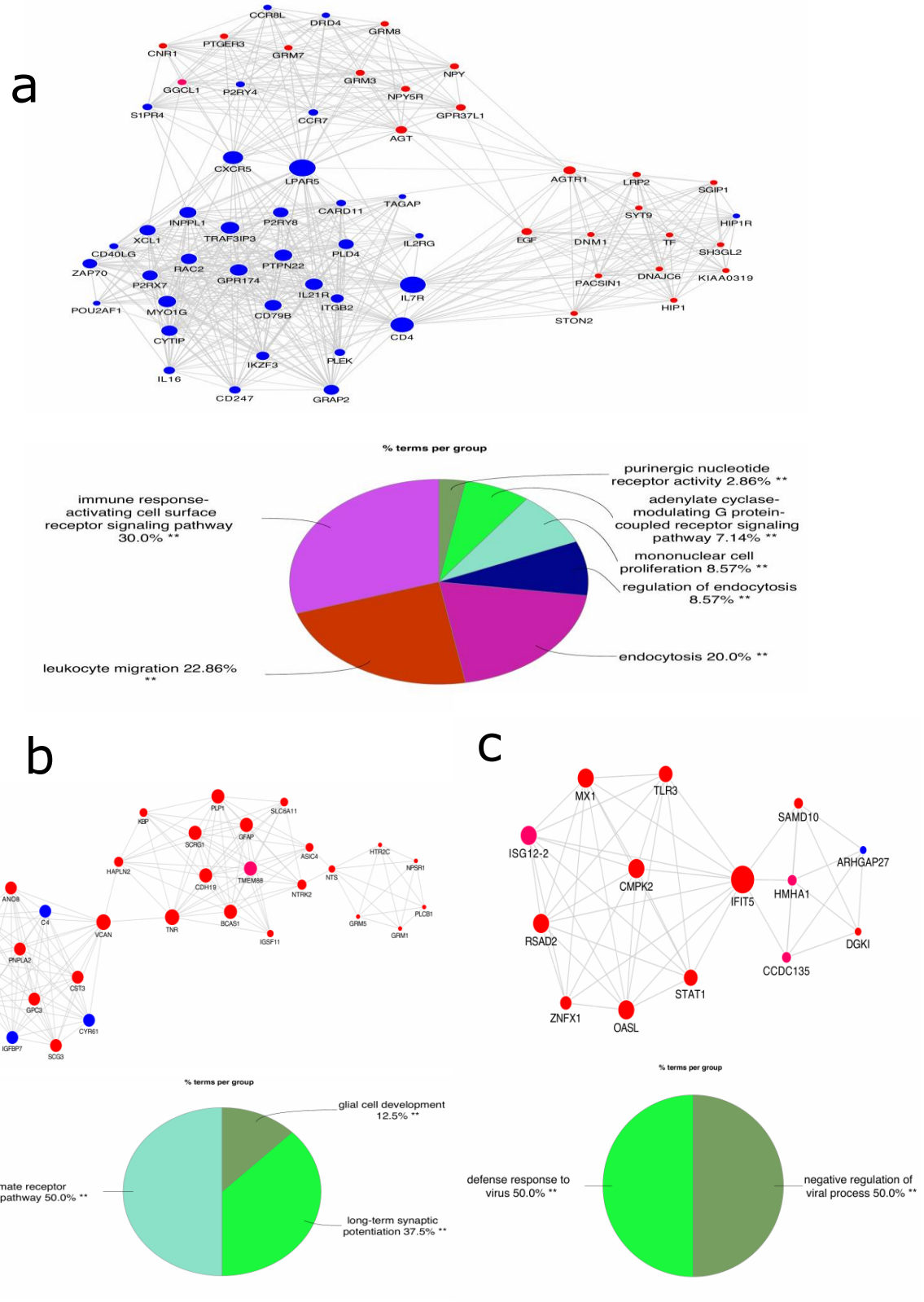
a
CCR8L
DRD4
GRM8
CNR1
PTGER3
GRM7
NPY
GGCL1
P2RY4
GRM3
NPY5R
S1PR4
CCR7
GPR37L1
AGT
CXCR5
LPAR5
AGTR1
LRP2
SGIP1
CARD11
TAGAP
SYT9
HIP1R
INPPL1
P2RY8
XCL1
TRAF3IP3
EGF
DNM1
TF
SH3GL2
IL2RG
CD40LG
PLD4
RAC2
PTPN22
ZAP70
GPR174
DNAJC6
KIAA0319
P2RX7
IL21R
IL7R
PACSIN1
POU2AF1
MYO1G
ITGB2
HIP1
CD79B
STON2
CD4
CYTIP
IKZF3
PLEK
IL16
CD247
GRAP2
% terms per group
purinergic nucleotide receptor activity 2.86% **
adenylate cyclase-modulating G protein-coupled receptor signaling pathway 7.14% **
mononuclear cell proliferation 8.57% **
regulation of endocytosis 8.57% **
endocytosis 20.0% **
leukocyte migration 22.86% **
immune response-activating cell surface receptor signaling pathway 30.0% **
b
PLP1
SLC6A11
KBP
SCRG1
GFAP
ASIC4
HTR2C
NPSR1
HAPLN2
CDH19
TMEM88
NTS
NTRK2
ANO8
C4
FAM20C
VCAN
TNR
BCAS1
GRM5
PLCB1
IGSF11
GRM1
PNPLA2
LAMB1
CST3
SCG2
GPC3
CYR61
IGFBP7
SCG3
% terms per group
glial cell development 12.5% **
glutamate receptor signaling pathway 50.0% **
long-term synaptic potentiation 37.5% **
c
MX1
TLR3
SAMD10
ISG12-2
ARHGAP27
CMPK2
IFIT5
HMHA1
RSAD2
DGKI
CCDC135
STAT1
ZNFX1
OASL
% terms per group
defense response to virus 50.0% **
negative regulation of viral process 50.0% **

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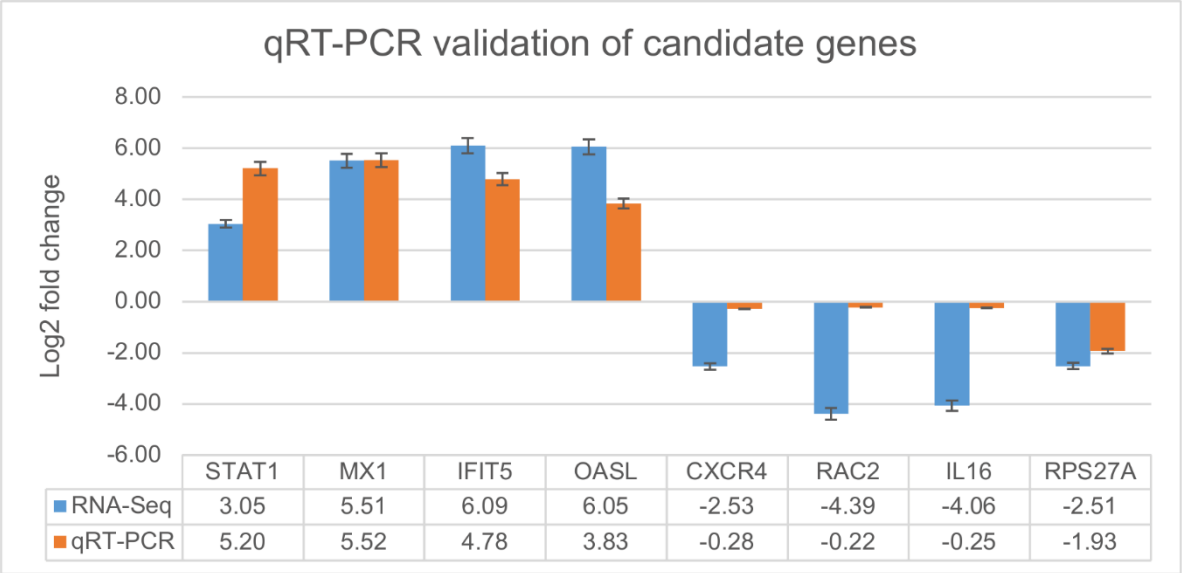
Fig 3.8. The top 3 modules of the PPI network and Gene Ontology annotation for genes in corresponding modules (a,b,c) shown in the pie charts. The red and blue node represent upregulated and downregulated genes in the data set, respectively. The size of the nodes illustrates the degree of connectivity. **P <0.001 shows the level of significance of the enriched terms. The chart fragments represent the number of genes associated with the terms as a proportion with the total number of genes within the GO term.

3.3.6 Validation of RNA-seq data using qRT-PCR

To further validate DEGs obtained from RNA-seq analysis, a total of eight DEGs associated with immune response were analysed by qRT-PCR. The log_2FC ratio for both RNA-Seq and qRT-PCR were compared, as shown in Fig 3.9. Linear regression analysis of showed a positive correlation (R = 0.91, P < 0.001).



	STAT1	MX1	IFIT5	OASL	CXCR4	RAC2	IL16	RPS27A
RNA-Seq	3.05	5.51	6.09	6.05	-2.53	-4.39	-4.06	-2.51
qRT-PCR	5.20	5.52	4.78	3.83	-0.28	-0.22	-0.25	-1.93

Fig 3.9. Validation of differentially expressed genes by RT-qRT-PCR. Log2 fold change as compared to the control group was normalizing the data to ACTB and GAPDH.

3.4 Discussion

NDV-GVII are strains of NDV cause marked tissue damage in lymphoid organs of infected birds (Elfatah et al. 2021; Susta et al. 2011; Wang, Y et al. 2012). In this study, histopathological examination revealed widespread lymphoid cell destruction in spleens of challenged birds (data not shown). Body and spleen weights were markedly reduced in infected birds compared to the control, and there was a significantly higher viral load in lymphoid tissues ($P<0.001$). Generally, weight loss is linked with increasing viral loads (Batterham, Garsia & Greenop 2002), and high viral load is strongly connected with increased disease severity and higher mortality (Fajnzylber et al. 2020), so these general features help to explain the rapid onset of severe ND by highly pathogenic NDV-GVII. The reduced number of lymphoid cells indicated the specific tropism of the NDV-GVII to the lymphatic tissues. Moreover, gastrointestinal, respiratory and neurological signs typical of other NDV strains were not observed; instead, there was a general hyperemia and significantly decreased size of spleen and bursa of Fabricius. The findings provide strong evidence that the virus can alter its tropism to lymphoid organs, and can become a systemic, lymphotropic virus rather than a gastrointestinal/respiratory virus.

We focused on the major pathogenic genes and pathways that help to explain the unique clinical, pathological and molecular findings of this newly emerged strain. To this end, RNA-seq was used to identify differentially expressed genes and pathways involved in the molecular pathogenesis and to help provide a comprehensive understanding of host response regulation under different conditions(Haq et al. 2010; Li, W et al. 2019; Nie et al. 2012; Wang, X et al. 2006). Our recently published paper also shows the association of apoptosis and necrosis with the pathogenesis of NDV-GVII (Rabiei et al. 2021). However, the disease production and destruction of lymphoid tissues by NDV-GVII could stem from multiple mechanisms. Therefore, this study aims to characterize the changes in the host transcriptome

profiles in chicken spleen to gain further insight into the modulation of the host immune responses upon infection with pathogenic NDV-GVII. Although the post-mortem findings were well supported by transcriptome analysis, some aspects of the phenotype, such as hemorrhages on the legs and breast muscle darkening remain to be elucidated. Perhaps these signs might be due to severe and generalized inflammatory responses and possibly consequent hypoxia or myoglobin degeneration(Maxwell, Robertson & Spence 1986). Dark breast muscle has been reported in chronic forms of Fowl Cholera (Pasteurellosis) (Herenda, Chambers & Ettriqui 1994), but never in NDV.

RNA-seq analysis indicated a downregulation of signaling pathways involving cellular immune response; cytokine signaling; cellular growth, proliferation and development; cellular stress and injury; and cell cycle regulation. Among the top twenty pathways, the most significantly inhibited pathway was fMLP signaling in Neutrophils. Neutrophils are not only key players in the inflammatory and innate immune responses against microbial pathogen invasion but also the major cause of tissue destruction. Upon the infection, neutrophils become activated through interaction with chemo attractants such as N-formyl-Met-Leu-Phe (fMLP), a chemoattractant playing a substantial role in larger scale necrotic damage, which trigger intracellular signaling transduction pathways, resulting in cell migration, phagocytosis, superoxide production, degranulation and so on (Mortaz et al. 2018). However, dysfunction of neutrophils and their hyperactivity is strongly involved in the pathogenesis of a wide range of inflammatory diseases, causing tissue damage (Peiseler & Kubes 2019). Therefore, the inhibition of fMLP (a chemoattractant playing a significant role in larger scale necrotic damage) signaling in neutrophils by NDV-GVII is highly likely associated with the observed lymphoid destruction.

This study also revealed significant downregulation of PI3*K* signalling in B Lymphocytes in response to the viral infection. Activation of PI3K signalling at the early stages of infection

has essential roles in viral replication, cell survival and proliferation in response to viruses such as avian influenza virus, NDV, and other paramyxoviruses (Guo, H et al. 2007; Kang et al. 2017). Sendai viral infection induces inhibition of PI3K signalling and results in faster apoptosis infected cells (Peters, Chattopadhyay & Sen 2008). As NDV-GVII is a paramyxovirus, the inhibitory effect of PI3K signalling may also be correlated with enhanced apoptosis. Our recently published paper showed that dysregulation of cell death related pathways is associated with the pathogenesis of the NDV-GVII. Furthermore, Fcγ receptor-mediated phagocytosis in macrophages and monocytes has been shown to be a significant contributor to clearance of bacterial and fungal infections and influenza virus infections(Huber et al. 2001; Ravetch & Clynes 1998; Yuan et al. 1998). The engagement of Fcγ receptors allowed opsonized microbes and immune complexes to initiate oxidant-sensitive signalling pathways that significantly increased host defence capabilities, but also promoted tissue damage(Pricop & Salmon 2002). The Leukocyte Extravasation signalling pathway involving inflammatory response was predicted to downregulate cell mobility and actin cytoskeletal contraction (Munir, Sharma & Kapur 2005). This inhibition also matches with a previous report that highly pathogenic NDV infection inhibited the actin-related cytoskeletal proteins. In contrast, NDV-La Sota strain induced the activation of actin cytoskeleton contraction and cell mobility in leukocyte extravasation signalling, suggesting a distinguishable mechanism of this virus (Deist, Melissa S et al. 2017). The substantial inhibition of immunologically regulated pathways may result in widespread immune suppression and could be one of the evolved strategies of the virus to evade the host immune response. Consistent with this observation, a recent study showed that NDV-GVII induces less effective immune responses and antiviral effects than NDV-La Sota strain that result from apoptotic and necrotic effects in CEFs (Zhao, J et al. 2019).

Virus-induced downregulation of host expressed genes has an important role in inhibiting the host antiviral responses (Lyles 2000). DEGs for the phosphoinositide 3-kinases (PI3K) complex are involved in most of the immune-related pathways and are responsible for activation of Akt/PKB which directly or indirectly affects CREB, consistent with previous transcriptome studies in chickens infected with NDV-La Sota (Zhan et al. 2020; Zhang, Jibin et al. 2018). Furthermore, genes in the NFkB transcription factor family that regulate cell proliferation, survival, differentiation, immunity, and inflammation (Oeckinghaus & Ghosh 2009) , and in the NFAT family that are involved in immune response (Rao, Luo & Hogan 1997) are also downregulated as part of most of the predicted pathways (Willard & Koochekpour 2013). Hence, the inhibition of PI3K, NFkB, and NFAT complex by NDV-GVII appears to have an inhibitory effect on cell survival, cell proliferation and host immune system function.

Pathway analysis alone may not be able to uncover the molecular basis of disease as it represents signaling, genetic, metabolic or neural processes in a linear fashion without considering protein interactions. PPI network analysis allows mapping of functional or physical interactions between gene product pairs that can have critical effects in complex diseases (Gonzalez & Kann 2012). PPI network analysis is, therefore, commonly used to identify molecules closely correlated with disease (Oti et al. 2006), and to identify disease-related subnetworks and study network properties (Sevimoglu, Tuba & Arga, Kazim Yalcin 2014). PPI network analysis revealed the top three gene modules that were closely related to disease progression. Functional enrichment analysis of subnetworks indicated that the first and third modules were associated with an immune-response activating cell-surface receptor signaling pathway, leukocyte migration, endocytosis, defense response to the virus and negative regulation of viral process. The activation of several interferon-inducible proteins identified in the third module is known to suppress the NDV replication, consistent with the

previous studies that virulent NDV induces strong innate immune responses (Ecco et al. 2011; Hu, Zenglei et al. 2015; Rue et al. 2011; Zhang, Jibin et al. 2018). However, over-activation of IFNγ cytokine in response to the virus also has a role in the activation of macrophages that can damage normal host tissues. The second module was enriched in glutamate receptor signaling pathway, long-term synaptic potentiation, and glial cell development. This finding is consistent with our previous work showing that the virus triggers a significant upregulation of neurotransmitters signaling pathways that can contribute to the apoptosis of neurons (Rabiei et al. 2021). Overexpression of these glutamate receptors and other neurotransmitters signaling pathways could cause the excitotoxicity and neuronal cell death that contribute to disease severity (Domercq, Vázquez-Villoldo & Matute 2013; Willard & Koochekpour 2013).

Furthermore, eleven novel hub genes (EGF, LPAR5, AGT, AGTR1, RAC2, CD4, CD3D, IL7R, NPY, GRM3, and GRAP2) were identified by PPI network analysis. EGF protein and its receptor EGFR play a crucial role in cell proliferation and survival, motility, angiogenesis, and endocytosis. EGF acts by binding EGFR, altering EGFR signalling in a way that facilitates the viral entry, replication, and inflammation, impairing the antiviral activity of IFN and antagonizing the host immune response (Lupberger et al. 2013; Zheng, Kitazato & Wang 2014). Moreover, EGFR has been shown to be activated by Respiratory Syncytial Virus (RSV) infection resulting in increased inflammation and delayed apoptosis (Monick et al. 2005). NDV and RSV are from the same viral family and may act via similar molecular pathways to cause disease. Lysophosphatidic acid (LPA) signalling via LPAR5 triggers microglial migration response and promotes a pro-inflammatory microglial phenotype (Plastira et al. 2017), whereas LPAR5 is an inhibitory receptor involving in negatively regulating TCR signalling, BCR signalling, B cell activation, and immune cell function (Hu, J et al. 2014; Mathew et al. 2019). An immune response can be mounted more effectively by

enhanced activation of a wide range of CD8 T cells through LPAR5 signalling in lymphoid tissues (Mathew et al. 2019).

AGT and its receptor AGTR1 have been shown to regulate T cell function and immune responses of the different diseases (Nataraj et al. 1999; Silva-Filho et al. 2011). While AGT is a pro-inflammatory mediator and participates in the recruitment of inflammatory cells into the tissues, overexpression of AGT and AGTR1 may result in tissue injury and contribute to disease severity (Ruiz-Ortega et al. 2001). RAC2 is a crucial regulator of the neutrophil actin cytoskeleton, cell migration, and the NADPH oxidase, and the dysregulation of RAC2 appears to lead to severe neutrophil dysfunction and a predisposition to the infection (Ambruso et al. 2000). Deletion of both RAC1 and RAC2 reduced CD4 and CD8 populations in the spleen and impaired proliferation of the splenocytes in response to TCR activation and B cell development (Guo, F et al. 2008; Walmsley et al. 2003). CD4 has a pivotal role in the immune response, and is also a coreceptor for MHC class II in T cells, promoting cytokine production and cell migration (Doyle & Strominger 1987; Zhen et al. 2014). CD4 protein is downregulated by HIV - Nef and Vpu viral proteins through the intensive endocytosis by a Clathrin/AP2 pathway, which is essential for entry, replication in T lymphocytes, and viral spread (Lindwasser, Chaudhuri & Bonifacino 2007; Lundquist et al. 2002), so the inhibition of host gene CD4 by NDV-GVII is likely to increase NDV virulence in a manner similar to HIV. CD3D is part of the TCR/CD3 receptors on the T lymphocyte cell surface, essential for T cell development and signal transduction (Dadi, Simon & Roifman 2003). The deficiency of CD3D can cause severe combined immunodeficiency (de Saint Basile et al. 2004). IL7R is a receptor for IL7 that has a pivotal role in T cell survival and homeostasis, promoting T cell proliferation and preventing apoptosis mainly through the Janus kinase signal transducer and activator of transcription phosphoinositide 3-kinase (PIK3) signalling pathway (Park, JH et al. 2004; Takada & Jameson 2009).

NPY, a neurotransmitter peptide, has a role in modulating cytokine production and secretion, natural killer cell activity, phagocytosis, T helper cell differentiation, and chemotaxis of the immune cells (Dimitrijević & Stanojević 2013; Ferreira et al. 2012). Moreover, NPY also plays a role in neuro-inflammation, neuron proliferation, and neuronal survival (Li, C et al. 2019). This suggests that NPY overexpression may be strongly associated with the host protective response to the insult produced by neurovirulent NDV- GVII infection. GRM3 is a G-protein-coupled receptor that regulates the induction of the inflammatory phenotype in microglial cells, cell migration, cell proliferation, and glutamate release (Crupi, Impellizzeri & Cuzzocrea 2019). This receptor's activation may counteract glutamate release and decrease excitotoxicity (Bratek et al. 2018; Crupi, Impellizzeri & Cuzzocrea 2019). GRAP2 promotes the activation of NFAT in T cells and reorganizes the cytoskeleton during T cell activation by interacting with SLP -76 leukocyte protein (Law, CL et al. 1999), while GRAP2 expression in human B cells also enhances B cell antigen receptor signalling crucial for the cell development and survival of B lymphocytes (Yankee et al. 2003). Among these novel hub genes, LPAR5, RAC2, CD4, CD3D, IL7R, and GRAP2 were significantly downregulated, whistle EGF, AGT, AGTR1, NPY and GRM3 were up-regulated in response to NDV-GVII infection. The overexpression of these novel genes may be correlated to the lymphocyte depletion and disease progression.

In conclusion, comprehensive bioinformatic analysis of genes induced by virulent NDV-GVII provides a deeper understanding of the host-pathogen interactions and sheds light on NDV-GVII pathogenesis at the molecular level. By targeting the lymphocytes and destroying these cells, NDV-GVII triggers a strong downregulation of immunologically regulated pathways such as fMLP signaling in neutrophils, *PI*3K signaling in B Lymphocytes and Fcγ receptor-mediated phagocytosis in macrophages and monocytes etc., which limit the host immune responses and antiviral effects and increases viral replication. Novel disease and

biological functions related subnetworks including immune response-activating cell surface receptor signaling pathway, leukocyte migration, endocytosis, glutamate receptor signaling pathway, defense response to the virus and negative regulation of viral process were identified. This present study also reported novel genes EGF, LPAR5, AGT, AGTR1, RAC2, CD4, CD3D, IL7R, NPY, GRM3, and GRAP2, which may be potential biomarkers for the molecular pathogenesis of virulent NDV-GVII. This study has provided comprehensive information about the NDV-GVII- induced destructive effects on the host immune system, but analysis of gene expression in other tissues is required to confirm and extend the identified pathways and hub genes.

Author Contributions: P.T.K.D**:** experiment work, analysing, and interpreting data, writing, reviewing and editing the manuscript, acting as the corresponding author; W.L: RNA-seq pipeline, reviewing and editing paper; Y.R: data analysis and reviewing paper; R.T: supervising, reviewing and editing paper; P.P: experiment work and reviewing; M.R: experiment work; F.H: conceptualising, supervising, funding acquisition, investigating. All authors have read and agreed to the published version of the manuscript.

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Data availability. Gene expression data were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA675698

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Conflicts of Interest: The authors declare no conflict of interest

CHAPTER 4: NEWCASTLE DISEASE VIRUS GENOTYPE VII GENE EXPRESSION

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Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Phuong Thi Kim Doan		
Contribution to the Paper	Doing experimental work, analysing, and interpreting data, writing, reviewing and editing the manuscript, acting as the corresponding author		
Overall percentage (%)	65%		
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	20.12.2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

vii. the candidate's stated contribution to the publication is accurate (as detailed above);
viii. permission is granted for the candidate in include the publication in the thesis; and
ix. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Newcastle disease virus genotype VII gene expression in experientially infected birds

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Abstract

Newcastle disease virus genotype VII **(**NDV-GVII) is a highly contagious pathogen responsible for pandemics that have caused devastating economic losses in the poultry industry. Several features in the transcription of NDV mRNA, including differentially expressed genes across the viral genome, are shared with that for other single, non-segmented, negative-strand viruses. Previous studies measuring viral gene expression using northern blotting indicated that the NDV transcription produced non-equimolar levels of viral mRNAs. However, high throughput sequencing (HTS) of virus-infected tissues can provide a better insight into the patterns of viral transcription. In this report, the transcription pattern of virulent NDV-GVII was analysed using HTS and qRT-PCR. This study revealed the transcriptional profiling of this highly pathogenic NDV-GVII genes: NP:P:M:F:HN:L, in which there were a slight attenuation at the NP:P and HN:L gene boundaries. Our result also provides a fully comprehensive qRT-PCR protocol for measuring viral transcript abundance that may be more convenient for laboratories where accessing HTS is not feasible.

4.1 Introduction

Newcastle disease virus is a member of the *Avian orthoavulavirus 1* species in the genus *Orthoavulavirus* subfamily A*vulavirinae,* and family *Paramyxoviridae* (https://talk.ictvonline.org/ictv-reports/). NDV strains possess a single-stranded, non-segmented and negative-sense RNA genome that is approximately 15,000 nucleotides in length. The genome contains six major genes encoding six proteins: nucleocapsid protein (NP); phosphoprotein (P); matrix protein (M), fusion protein (F); hemagglutinin protein (HN) and large protein (L) in the order 3'-NP-P-M-F-HN-L-5'. Additionally, two non-structural proteins V and W are derived from P (Swayne & Glisson 2013; Yusoff & Tan 2001). HN and F encode two surface glycoproteins and are responsible for virus entry and budding, respectively. M protein is required for the integrity of the virus particles and is located on the inner surface of the envelope, whereas NP, P and L proteins constitute a viral RNA-dependent RNA polymerase (RdRP) complex that has a crucial role in RNA transcription and synthesis (Whelan, Barr & Wertz 2004; Yusoff & Tan 2001). In addition, V protein modulates the viral RNA replication through inhibition of host IFN signalling (Ganar et al. 2014).

Like other paramyxoviruses, NDV uses its negative-sense genome as the template for the viral RNA transcription to generate six separate viral transcripts (Conzelmann 1998; Whelan, Barr & Wertz 2004). The 3' and 5' end of the genome contains extra-cistronic regions of 55nt and 114nt which make up the 3' leader and 5' trailer region, respectively. Transcription occurs when RdRP recognises and attaches to the leader promoter and scans along the genome until it reaches the first gene start (GS) site, where NP gene transcription is initiated. It appears that capping and methylation are carried out by RdRP via a signal from the GS site and polyadenylation occurs after the gene end (GE) site (Whelan, Barr & Wertz 2004; Wignall-Fleming et al. 2019). The 5' capped, methylated and 3' polyadenylated viral

transcript is then released by the RdRP. After terminating the transcription of an upstream mRNAs, RdRP either disengages from the genome at a GE site and reinitiates at the leader promotor to continue transcribing or traverses the intergenic region and reinitiates at a GS site of the next gene to transcribe. The viral mRNAs of negative strand RNA viruses are most likely synthesized by a sequential and discontinuous mechanism involving polymerase stop-start signals, which terminate at the end of each preceding gene and restart at the beginning of the next gene (Abraham & Banerjee 1976; Cattaneo, Rebmann, Schmid, et al. 1987; Collins, Hightower & Ball 1978; Whelan, Barr & Wertz 2004). Because not all RdRP reinitiate, the mRNA is more abundant for genes nearer the 3' end compared with those nearer the 5' end of the viral genome (Noton & Fearns 2015), a phenomenon known as a transcriptional gradient(Yusoff & Tan 2001).

However, this process is not always efficient and RdRP occasionally fails to terminate the transcription at the gene-end site, which leads to the transcription of mRNA across the intergenic region and downstream gene(s), producing different loads of mRNA (Homann, Hofschneider & Neubert 1990; Wright, Crameri & Eaton 2005). The viral gene expression of several paramyxoviruses such as Measles, Hendra, Sendai, Mumps virus and Parainfluenza virus type 2, 3, 5 have been studied (Cattaneo, Rebmann, Baczko, et al. 1987; Homann, Hofschneider & Neubert 1990; Wignall-Fleming et al. 2019; Wright, Crameri & Eaton 2005). Transcriptional gradients of viral mRNAs and steep attenuation of transcription occur at M-F and G-L junctions of Hendra virus, at M-F and HN-L junctions of Sendai virus and at NP-P and HN-L junctions of Measles virus. Furthermore, the transcription attenuation varies between different strains of vesicular stomatitis virus (VSV), another non-segmented, negative-strand RNA virus (Hodges, Heinrich & Connor 2012). An isolate with a small plaque phenotype was found to have a steeper transcription gradient and to generate fewer mRNA transcripts than the wild-type virus (Hodges, Heinrich & Connor 2012). Similarly, the

transcription patterns of the respiratory syncytial and Ebola viruses is genotype dependent, but was in an non-transcriptional gradient pattern (Pagán, Holmes & Simon-Loriere 2012; Piedra et al. 2020), suggesting gene expression mechanisms of pathogenic viruses are variable.

Non-equimolar amounts of NDV polypeptides are produced both in vitro and in vivo (Collins, Hightower & Ball 1978). However, the quantification of viral transcripts using Northern blotting appears to be relatively less insensitive, so high throughput sequencing (HTS) has been used to measure the viral mRNAs after viral infection (Albariño et al. 2018; Wignall-Fleming et al. 2019). Using counts per million of viral transcripts as the measure, there is a gradual decrease in amounts from the 3' to 5' end of the genome in the trachea epithelial cells (Deist, Melissa S et al. 2017). In contrast, there were higher viral mRNA amounts of F and HN genes found in the Harderian gland of challenged birds. In addition, the quantification of viral transcripts in experimentally challenged birds with La Sota strain revealed that the abundance of mRNAs was significantly higher in Trachea epithelial cells than those in Harderian gland of the birds. These studies suggest that the host genome can also modulate differential gene expressions of viruses.

It is generally accepted that the F protein cleavage site is a major molecular determinant of NDV virulence (Peeters, BP et al. 1999). Despite sharing the similar F cleavage site associated with the high virulence, virulent strains of distinct genotypes trigger markedly different pathological signs and manifestations, especially in lymphoid organs (Ecco et al. 2011; Susta et al. 2011; Wang, Y et al. 2012). There may be other molecular determinants apart from F protein cleavage site related to virulence and pathogenicity of NDV-GVII strains. F and HN genes of highly virulent NDV CA02 strain were a determinant of macrophage tropism (Cornax, Ingrid et al. 2013). Additionally, Kai *et al.* shown that M, F and HN genes were related to the substantial replication of virus and strong innate immune

response, contributing to the severe tissue damage and pathogenic changes(Kai et al. 2015). Despite of significant differences in viral mRNAs transcription in different viruses, understanding of viral gene expression profiling of paramyxoviruses will help us to understand the disease production mechanisms in NDV-GVII in chickens. Few publications exist in virus-host interaction mechanisms, but they mainly focussed on very specific pathways using Northern blot, qRT-PCR and HTS in Sendai virus, Hendra virus or avirulent NDV (Albariño et al. 2018; Baczko et al. 1984; Wignall-Fleming et al. 2019; Wright, Crameri & Eaton 2005). This study utilised RNA-seq and qRT-PCR to identify the role of other viral genes that may contribute to pathogenicity by measuring viral mRNA genes in the infected tissue. NDV-GVII pathogenesis differs to that of other NDV genotypes, it is no information available on the viral gene expression of this highly pathogenic virus. This is the first study on viral gene expression on an experimental infection in live chicken.

4.2 Results

After infection with virulent NDV-GVII Mega strain, spleen tissues were harvested, the RNA extracted, and reverse transcribed to cDNA. Six pairs of primers for NP, P, M, F, HN, and L were designed to amplify NDV gene fragments specifically. Resulting PCR products of the expected size were cloned into TOPO TA pCR 2.1vector and transformed into the chemically competent DH5 alpha strain of E. *coli* Cells (ThermoFisher Scientifics, VIC, Australia). Plasmids were then isolated, linearized and used to generate standard curves in absolute quantitative PCR methods. mRNA abundances (copy number) were converted from Ct values using created standard curves for each virus.

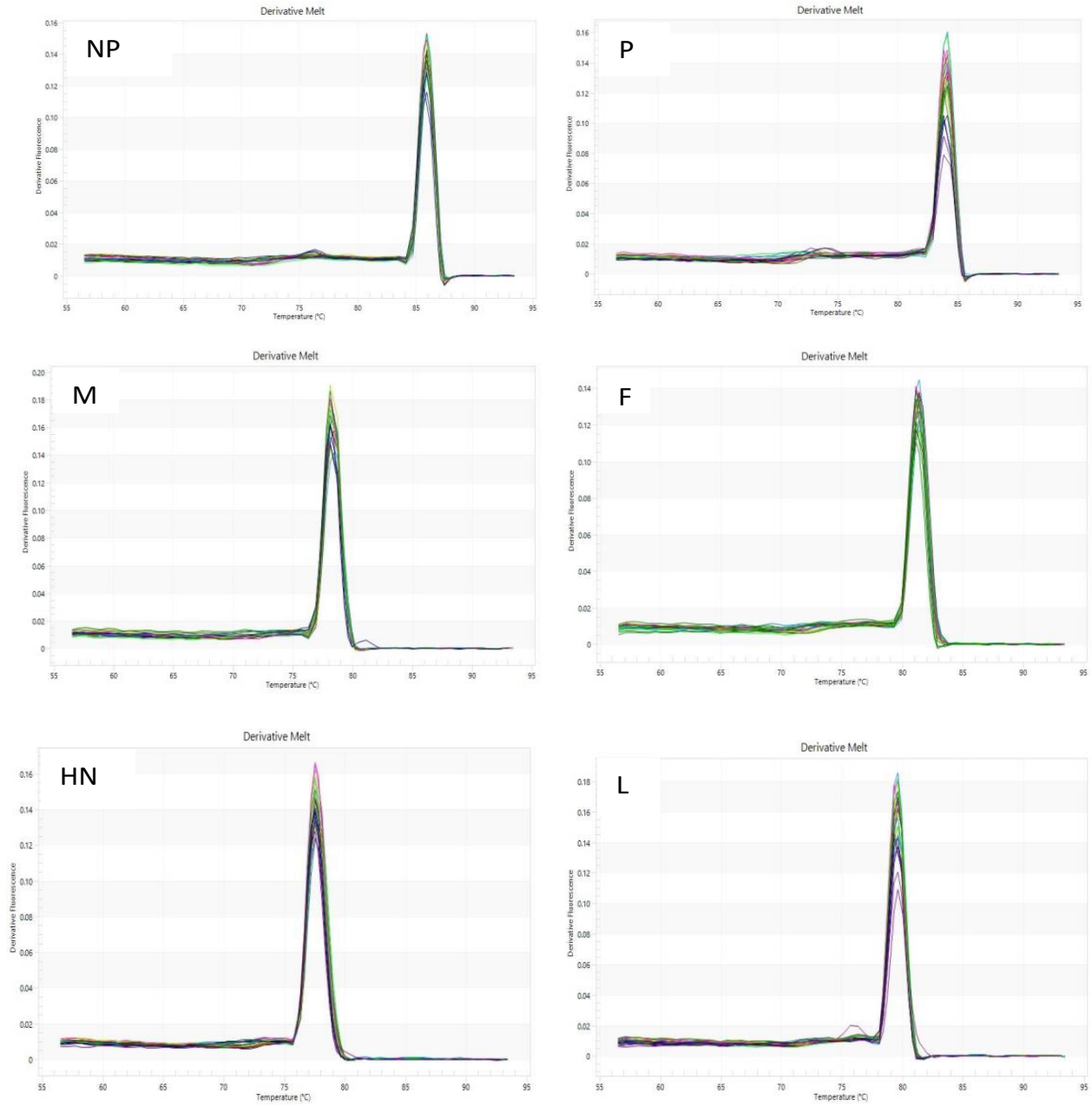
4.2.1 Primer optimising and specificity of the PCR

NP, P, M, F, HN, and L specific primers were used to amplify PCR products of 112bp, 123bp, 112bp, 91bp, 86bp and 128bp, respectively. The annealing temperature for each primer pairs is shown in Table 4.1.

Table 4.1 Primer pairs and PCR efficiencies used for RT-PCR and qRT-PCR

Gene symbol	Primer Sequence	Fragment size (bp)	Ta °C	Efficiency
NP	Forward: ATGAGAGCAGTGGCGAACAG Reverse: CCCAGTCAGTGTCGTTGTCT	112	60	1.94
P	Forward: CATCCTTAAGTGATCTCCGA Reverse: CCGGTTGTGAGAGTTTATTG	123	54	1.99
M	Forward: CTGCATATCGGGCTTATGTCCACT Reverse: GCACATCACTGAGCCCAACAGATA	112	62	1.93
F	Forward: AAGCTCTCTTGATGGCAGGC Reverse: CCCTGTTTGAGACGAGGTGT	91	58	1.99
HN	Forward: GGACATCTGCAACAGGGAGG Reverse: CACACTGCAGGACTTCCGAT	86	61	1.93
L	Forward: GCATCCACTGTAGCACGACTATGT Reverse: GGTTGCGAGCTGTGGGTAATAGAA	128	62	1.98

Melting curves for each PCR reaction were generated to test the specificity of the qRT-PCR reactions. Single melting curve peaks indicated that only target sequences were amplified (Fig 4.1).



975

976 Fig 4.1 Melting curve analysis for each PCR reaction of 6 target genes

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4.2.2 Standard curves and qRT-PCR amplification efficiency

Colony PCR run was performed to screen transformed bacteria with cDNA as a positive control and water as a negative control. The presence of the insert was confirmed by Sanger sequencing using M13 and NDV specific gene primers. Standard curves were generated by SYBR Green PCR for each primer pairs using 10-fold dilutions of purified and linearised plasmids in triplicate reactions, with the PCR amplification efficiencies between 93 and 99 % and correlation coefficients greater than 0.993 (Table 4.1 and Fig 4.2).

4.2.3 NDV mRNA abundances by RNA-seq and qRT-PCR analysis

NDV-GVII strains are more lymphotropic than other strains, so the level of NDV expression in spleens was analysed. To determine the relative abundance of individual viral transcripts, RNA-seq of spleen tissue was carried out and gene expression quantified using FPKM (Fragments per Kilobase of transcript Per million Mapped reads) as the measure. FPKM adjusts counts for gene length so expression can be compared between genes.

qRT-PCR was also used to validate RNA-seq data. Ct values for mRNA targets (NP, P, M, F, HN, L) were measured and then converted to mRNA abundance using standard curves of known concentration. Quantification of qRT-PCR was consistent, as shown by amplification efficiencies between 93 and 99% and high correlation between replicates ($R^2 = 0.99$). We used viral transcript amounts measured by RNA-seq and qRT-PCR to determine what percentage each viral mRNA contributed to the total viral mRNA (Fig 3). RNA-seq and qRT-PCR showed a high positive correlation using linear regression ($R = 0.96$, $P < 0.002$), confirming the RNA-seq data's reliability. Overall, there is a progressive decline in expression of each amplicon from the 3’ to the 5’ of the genome. NP mRNAs were most abundant contributing nearly 40% of total mRNAs and gradually decreasing thereafter. Normalising to the level of NP transcript, the level of gene expression was 100:69:51:31:29:6 for NP:P:M: F:HN: L, respectively.

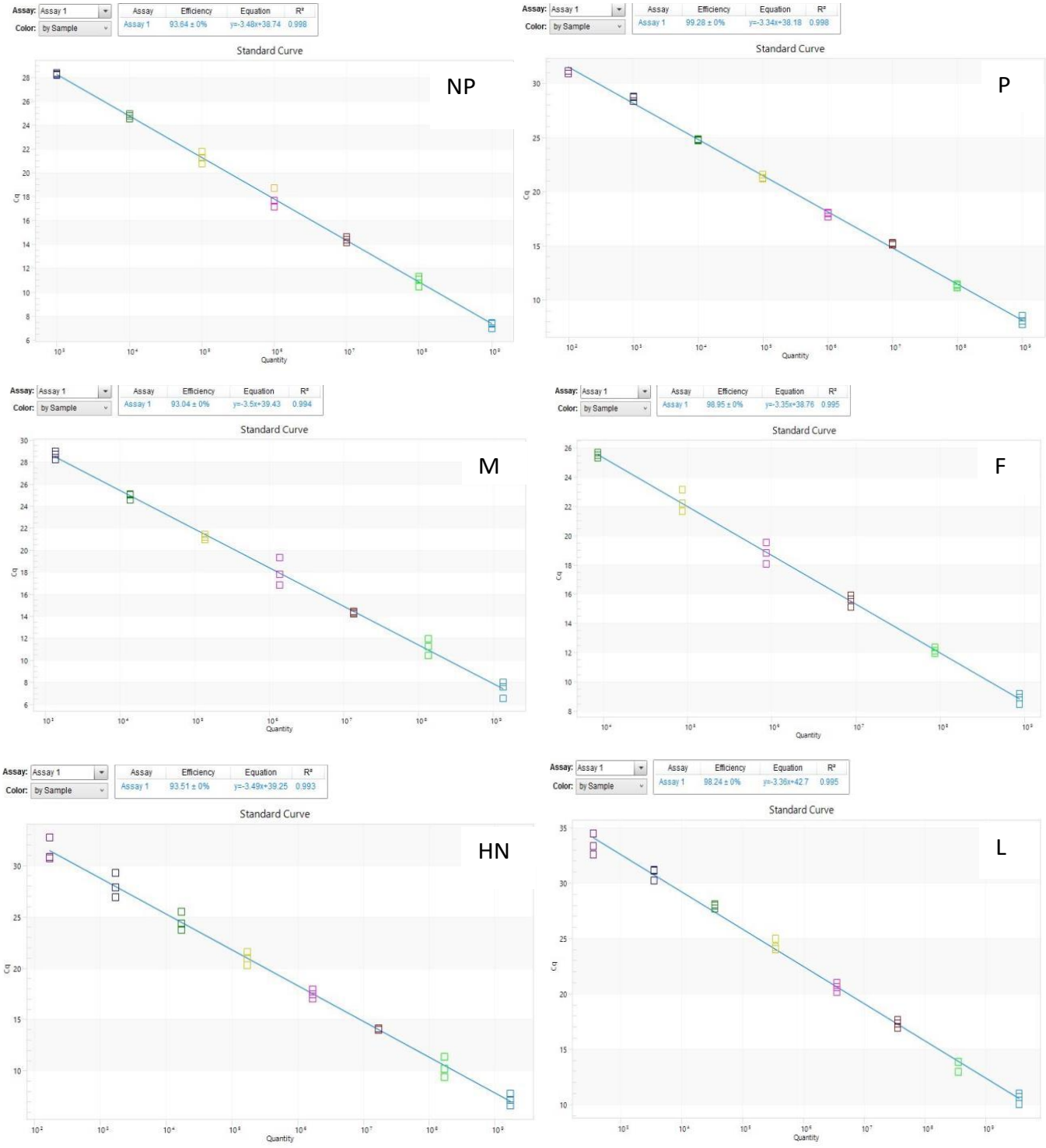

Fig 4.2. Standard curves of serially diluted linearised plasmids obtained using SYBR Green for each target sequence in order.

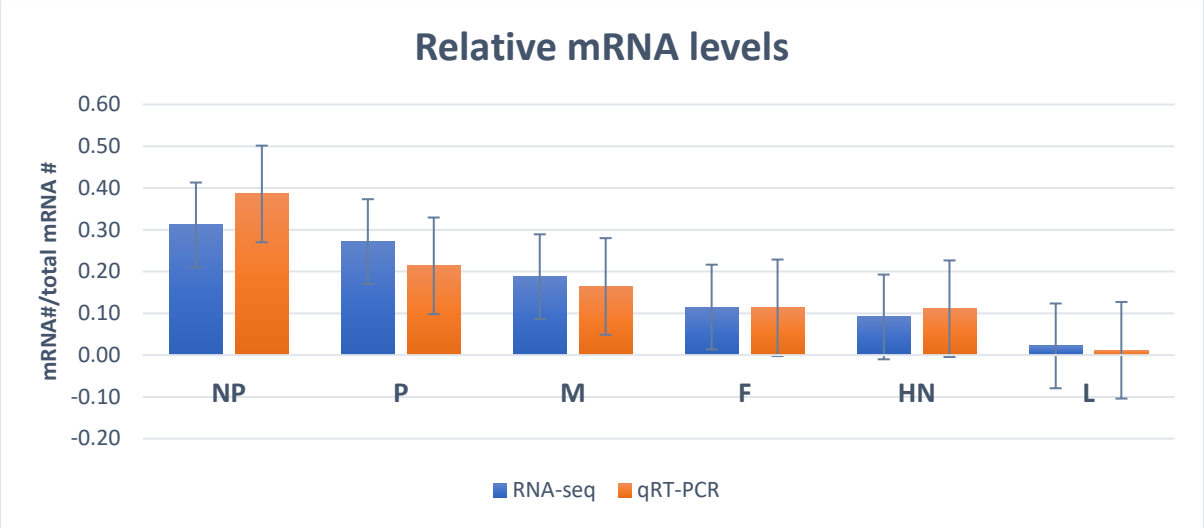

Fig 4.3. Histogram depicts the relative mRNA abundance gradients of viral genes from RNA-seq and qRT-PCR. Each bar describes individual mRNA molecules/ total mRNA molecules, and the error bars show the standard deviations of the means.

4.3 Discussion

Despite having the same virulent fusion protein cleavage site and high ICPI values associated with increased virulence, the pathogenicity of the NDV strains are quite dissimilar (Merino et al. 2011; Wang, Y et al. 2012). This suggests that there are elements of the viral genome other than the fusion protein cleavage site that play a role in the pathogenicity of NDV-GVII. One recent study revealed that the severe pathology of NDV-GVII was in part due to the high level of virus replication and potent inflammatory response driven by the M, F and HN genes (Kai et al. 2015) . Furthermore, F and HN genes contribute to macrophages tropism of virulent NDV (Cornax, Ingrid et al. 2013). However, the varying role of viral mRNA expression in the virulence of NDV-GVII remains unknown. We describe the differential expression of individual viral genes of NDV-GVII in experimentally infected birds, which may be correlated with the pathogenicity of the disease and may impact on tissue response to the virus using RNA-seq and qRT-PCR.

This study the abundance of different viral mRNAs by mapping RNA-seq data to the NDV-GVII genome, separating transcripts by open reading frame and normalising counts of gene reads by viral gene length (Fig 3). NP mRNA abundance was greater than that of L transcript, and there was slight attenuations at the NP:P and HN:L boundaries. The differential expression values of individual genes were confirmed by the absolute quantification method. Although the V gene was not specifically measured as part of gene expression, it was assumed that viral transcript counts of V gene would be expressed in a similar abundance to the P protein mRNA. Furthermore, it has been shown that transcriptional profiles of viruses vary depending on viral genotypes and host tissue. High-throughput sequencing as employed in the most recent study to investigate the paramyxovirus transcription(Wignall-Fleming et al. 2019) emphasized that the transcriptional profiles between PIV2, PIV3, PIV5 and mumps virus were substantially different, with NP highest in all except Mumps virus, PIV2 and PIV5

show a steep decline at the NP:V/P boundary, PIV3 and PIV5 at the HN:L boundary, and Mumps at the V/P:M boundary. In addition, while the NP viral transcripts for PIV2 and PIV5 are abundant, Mumps shows the same number of transcripts at NP and V/P. These suggest that NDV-GVII transcription is more similar to PIV5 than other paramyxoviruses.

The transcription of non-segmented negative, single-stranded RNA virus genomes is gradually attenuated along the genome and with the highest number of viral transcripts for gene near the 3' end and fewest for genes near the 5' end (Martínez et al. 2008; Whelan, Barr & Wertz 2004). However, the viral transcript abundance for the respiratory syncytial virus (RSV) and Hendra virus (*Mononegavirales)* is not necessarily correlated with the position of a gene in the viral genome (Aljabr et al. 2016; Piedra et al. 2020). The transcription of the NDV genome has previously been shown to produce transcripts in molar ratios for NP:M:F:HN:L of 100:41:65:22:3 (Collins, Hightower & Ball 1978). While the P viral polypeptide was not detected in the previous work and why the P protein is absent was not explored, the latter *in-vivo* experiment by these authors showed that the transcriptional pattern of NDV genome followed the NP: P: F: M: HN: L order (Collins, Hightower & Ball 1980). This may suggest a lack of the sensitivity and accuracy of this technique to quantify mRNA species. Another study used qRT-PCR to measure viral mRNA accumulation in cells infected with Hendra virus and found that there was a transcriptional gradient with sharp reductions in transcript abundance at the M:F and G:L boundaries (Wright, Crameri & Eaton 2005). However, RNA-seq data revealed a significant attenuation in mRNA abundance at the M:F gene junction only (Wynne et al. 2014).

Estimation of NDV transcript abundance after infection with an avirulent NDV strain (La Sota) revealed that individual gene expression levels were not reduced from the 3' to 5' end of the genome(Deist et al. 2018). In another recent study using qRT-PCR for gene expression quantification, illustrated that transcription of RSV (paramyxovirus) also depends

on the genotype of the viruses and non-gradient patterns (Piedra et al. 2020) that are inconsistent with what is known about the expressed mRNA levels when the genome of RNA virus transcribed (Cattaneo, Rebmann, Baczko, et al. 1987; Hodges, Heinrich & Connor 2012; Homann, Hofschneider & Neubert 1990).

Notably, the viral replication complex (NP, P, L) appears to be the major element of virus transcription that is associated with the gradient. Dorman *et al.* showed the significant contribution of NP, P, L in NDV virulence (Dortmans, J. C. et al. 2010). Moreover, in molecular evolution of *Mononegavirales*, gene expression level indicated as a major evolutionary determinant and pathogenicity for all of these viruses (Pagán, Holmes & Simon-Loriere 2012). Several studies on the evolutionary effects of recombinant variants of Rabies virus and vesicular stomatitis virus revealed that the expression level of outlier proteins P and G was not a major determinant, whereas viral replication was strongly correlated with the expression level of the N and M genes(Ball et al. 1999; Novella, Ball & Wertz 2004; Wertz, Perepelitsa & Ball 1998; Wirblich & Schnell 2011). The viral NP protein interacts with host translation machinery during NDV infection, activating multiple signalling pathways, especially PI3K/Akt/mTOR and p38MAPK/Mnk1 pathway for selective viral protein synthesis infected cells, which facilitate viral mRNA translation (Zhan et al. 2020). Moreover, Cheng *et al.* also revealed expression of NDV NP and P proteins to be important in viral replication and virulence by inhibiting of autophagy (Cheng et al. 2016). Interestingly, our recently published paper indicated that autophagy-regulated cell death was one of the most significantly inhibited pathways by NDV-GVII infection (Rabiei et al. 2021), that may be associated with the highest expressed levels of NDV-GVII NP and P genes and increased virulence. The efficient replication of many viruses is influenced by the expression ratio of proteins within the viral replication complex (Noda et al. 2011). This suggests that

the potential roles of these genes and the different levels of viral gene expression could be a crucial evolutionary determinant of pathogenicity.

In conclusion, the use of high-throughput sequencing and qRT-PCR allowed us to quantitate viral mRNAs in tissues infected with virulent NDV-GVII. The different expression ratio of viral proteins may have an important role in the virulence and pathogenicity of NDV-GVII disease. While qRT-PCR is more laborious it is also convenient and easy set up in molecular laboratories, RNA-seq is more expensive and requires more bioinformatics expertise. To the best of our knowledge, this is the first study quantifying virulent NDV-GVII transcripts in vivo, revealing the differences in NDV-GVII gene expression. Further investigation is required to determine whether other genotypes of NDV have the same transcriptional pattern and whether these patterns can be associated with their pathogenicity.

4.4 Materials and Methods

4.4.1 Virus preparation and collection of experimentally infected tissues

NDV isolate Chicken/Indonesia/Mega/001WJ/2013, accession number MN688613.1(Doan et al. 2020). All animal experiment procedures at the Indonesian Research Centre for Veterinary Science (Bbalitvet), Bogor, Indonesia were approved by the ethics committee with the reference number of A.H./2015/003. According to the National Health and Medical Research Council of Australia guidelines, the experimental chickens were supervised and monitored by A specialized veterinarian. Twenty-one-day-old specific pathogen-free (SPF) layer chicks were reared at the Animal House Facility in the Indonesian Research Centre for Veterinary Science (Balitvet). A hemagglutination inhibition test was performed on the serum sample from each chicken to ensure no antibody against NDV exists in the birds. The birds were separated into two groups. The first group was a negative control and received no virus, while the second group was challenged with virulent NDV-GVII Mega strain. The birds were inoculated by intraocular and intranasal instillation with 100µL of 100 EID50 of live NDV-GVII at 35 days of age (Alexander, D. J., Manvell & Parsons 2006; Miller et al. 2013). PBS was used as the placebo for non-infected birds. The birds were monitored daily for clinical signs, morbidity, and mortality and were then bled via a brachial vein or by cardiac puncture at the terminal step just after euthanasia. On day 3 after challenge, all surviving birds were euthanized and necropsied to collect tissue samples for RNA isolation. Spleens were collected and placed into RNA later stored at -80°C for later use.

4.4.2 RNA extraction and cDNA synthesis

Total RNA from 50mg of spleen from each uninfected and infected bird (10 birds for each group) was extracted using a commercial *mir*Vana™ miRNA Isolation Kit (QIAGEN, California, USA) according to the manufacturer's instruction. These total RNAs were also

used to isolate mRNA for qRT-PCR. Poly(A)$^+$RNAs were selected from total RNA using ROCHE mRNA isolation kit (SigmaAldrich, Australia) according to the kit instruction. Two-step RT-PCR was used to quantify the viral mRNA transcripts for each gene. cDNA was synthesized using the QuantiTect Reverse Transcription kit (QIAGEN *GmbH*, Hilden, *Germany)*. The optimized blend of oligo-dT and random primers enables high cDNA yields from all regions of viral RNA transcripts. Reactions carried out in 20µl volumes in 0,2ml thin wall, flat cap PCR tubes were including 2 steps. Firstly, a mix of 2µl gDNA Wipeout Buffer 7x, 7µl of RNA free-water and 5µl of mRNA was prepared in tubes and incubated for 2 minutes at 42°C to effectively remove any gDNA contaminations then placed immediately on ice. Next, tubes of the reverse-transcription master mix containing 1µl of Quantiscript Reverse Transcriptase, 4µl Quantiscript RT Buffer 5x and 1µl RT primer Mix was prepared on ice. Then, template RNAs from the first step were added to each tube containing reverse-transcription master mix and heated to 42°C for 15 minutes and kept heating to 95°C for 3 minutes to inactivate Quantiscript Reverse Transcriptase. Finally, cDNAs were placed on ice before using or stored at -20°C.

4.4.3 mRNAs primer design, cloning, transformation of bacteria and plasmid isolation

Primers were designed for each viral gene (NP, P, M, F, HN, L) using the Primer-BLAST tool at NCBI. They were also aligned to the viral reference genome sequence to confirm that there were no mismatches, thus optimising accurate mRNA transcript quantification by qRT-PCR. The PCR products ranged from 60-150 base pairs, ideal for maximum PCR efficiency. Fresh PCR products corresponding to each of the six viral amplicons (NP, P, M, F, HN, L) were generated by PCR and TA cloned using the TOPO TA pCR 2.1 cloning vector kit (Invitrogene, California, USA) according to the manufacturer's instructions. The recombinant vectors were transformed into One Shot® Chemically Competent *E. coli* (Invitrogen) and 30µl of transformed culture was spread onto LB plates containing Ampicillin (1µl/ml).

Transformants, identified as white colonies, were selected and colony PCR performed on them using M13 primers and the corresponding gene-specific primers. Colonies were cultured overnight in LB medium containing 50µg/mL Ampicillin. Plasmid isolation was carried out using Gene Elute Plasmid Miniprep kit (Sigma-Aldrich). The presence of the insert in the recombinant clones was confirmed by sequencing using Sanger sequencing.

4.4.4 qRT-PCR verification of standard templates and standard curve construction

Plasmids carrying NP, P, M, F, HN, L inserts were linearised using the *HindIII* restriction site located upstream of the insertion site (Hou et al. 2010). Digestion products were then visualised by Gel electrophoresis to again confirm their size. DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The number of plasmid molecules were determined using the following website: https://cels.uri.edu/gsc/cndna.html. In brief, number of molecules = (plasmidamount * 6.022×10^{23}) / (plasmidlength * 1×10^{9} * 650). Optimal working concentrations were prepared for each plasmid; 1×10^{10} molecules/µl for NP; 1.7×10^{10} molecules/µl for P; 6.93×10^{9} molecules/µl for M; 8.93×10^{9} molecules/µl for F; 1.72×10^{10} molecules/µl for HN; and 7.13×10^{9} molecules/µl for L. Standard curves for each primer pair were generated by using at least six, 10-fold dilutions of the linearized plasmid. The qRT-PCR reaction was performed in 10µl, using 5µl 2X SYBR Green PCR Master Mix, forward primer (1µl, 10µM), reverse primer (1µl, 10µM), RNase-free water (1µl) and standard curves of linearised plasmid (2µl). The thermal cycling profile included a PCR initial heat activation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, and then combined annealing/ extension at 60°C for 30 seconds. To assess the specificity of amplification, a ramp of 60 - 99°C was added to the melting curve step and the specificity of the reaction was confirmed by melting curve analysis. All the reactions were carried out in triplicate, including negative controls without templates. The cycle threshold (Ct) values of each dilution were measured in

triplicate by The Eco real -time PCR system to generate the standard curves and were then plotted against the logarithm of the number of initial template molecules. Each standard curve was generated by a linear regression of the plotted points, creating the slope of each standard curve. PCR amplification efficiency were then calculated using the equation: $E = 10^{(-1/slope)} -1$ (Lee et al. 2006). Data acquisition was carried out and analysed by EcoStudy v.50.

4.4.5 qRT-PCR and NDV mRNA transcript measurements

The target sequences were amplified using the Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) as described. The specific primers for qRT-PCR were the same as those used in generating standard curves. cDNA was diluted into RNase-free water with a ratio of 1 in 10 before using in the PCR. All negative, positive, standard curves and tested samples for the selected primer sets were dispensed to 384 well plates using an automated Corbett robot system (Corbett Research, Sydney, Australia). Reactions were performed with Applied Biosystems Real-Time PCR instruments and thermo cycle profile as described above.

The *Ct* values of the samples for mRNA targets were converted to mRNA abundances using the standard curves. As described above, each dilution of standards was measured in triplicate *Ct* values and an average *Ct* was calculated. Standards were constructed by average *Ct* values and known amounts of each linearized plasmid (molecules/rxn). For cDNAs derived from spleen samples, *Ct* values and an average of each sample were measured in triplicate and calculated. Then each average *Ct* value of samples was converted to a viral transcript abundance using the linear relationship determined for the appropriate standard curve *Ct* versus logarithm of the standard amount.

4.4.6 High-throughput sequencing (HTS) and bioinformatic analyses

For each sample, 0.5 µg of total RNA was used to construct a cDNA library using an Illumina TrueSeq RNA sample preparation kit, according to the manufacturer's recommendation (Illumina. California U.S.A.). The library sequenced on an Illumina

NovaSeq S1 300 cycle instrument (Illumina. Inc, San Diego, CA, USA) to generate paired end reads of 150 bp length.

Raw RNA-seq reads were checked for quality using FASTQC v0.11.4 (Andrews 2010) and trimmed with TrimGalore v0.4.2 (Krueger & Galore 2015). Reads with a minimum length of 100 bp and where all the bases had a minimum Phred score of 10 were kept. AdapterRemoval v2.2.1 was used to eliminate adapter sequences and the reads were checked again with FASTQC (Schubert, Lindgreen & Orlando 2016). The cleaned reads were mapped to a combined chicken and virus reference genome containing the NDV-GVII Mega strain sequence and chicken reference genome (Gallus_gallus.GRCg6a), using Hisat2 v2.1.0 (Kim, D, Langmead & Salzberg 2015). Mapped reads were then sorted with SAMtools v1.8(Li, H et al. 2009) and were counted using R package featureCounts, with genes defined using Ensembl annotation version 97 (Liao, Smyth & Shi 2014). Fragments per kilobase of transcript per million mapped reads (FPKM) values were used to calculate relative mRNA abundances. FPKM values normalise viral mRNA amounts produced from each individual genes to account for gene length differences, thus, the relative abundance of viral transcripts from genes of different length can be compared.

Author Contributions: P.T.K.D**:** experiment work, analysing, and interpreting data, writing, reviewing and editing the manuscript, acting as the corresponding author; W.L: RNA-seq pipeline, reviewing and editing paper; Y.R: data analysis and reviewing paper; R.T: supervising, reviewing and editing paper; F.H: conceptualising, supervising, funding acquisition, investigating. All authors have read and agreed to the published version of the manuscript.

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Data availability. Gene expression data were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA675698

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Conflicts of Interest: The authors declare no conflict of interest

CHAPTER 5 GENERAL DISCUSSION

5.1 Introduction

Newcastle disease has significant negative impacts on the poultry industry, especially in nations where it is endemic such as Southeast Asia, Africa, the Middle East and some parts of Southern and Central America. Viruses such as NDV can be difficult to manage and contain in poultry farms because of their antigenic and genetic diversity in pathogenicity and a wide range of hosts. While lentogenic vaccine strains are often assumed to provide full protection against NDVs, highly virulent NDV strains, particularly NDV-GVII strains are the predominant pathogen in most recent NDV outbreaks, occurring even in heavily vaccinated chickens worldwide. Australia is currently considered free of pathogenic NDV, but NDV-GVII strains have been isolated from wild birds, posing a potential risk to Australian chicken industry (Elfatah et al. 2021; Turan et al. 2020). The main purpose of this dissertation has been to investigate the underlying molecular mechanisms of pathogenesis of NDV-GVII in chickens, including the first use of RNA-seq to analyse the chicken's spleen response to NDV-GVII in vivo. The first objective of this dissertation was to isolate and detect highly virulent NDV strains that have been responsible for ND outbreaks in vaccinated chicken farms. The findings indicated the prevalence of NDV-GVII strains in these recent outbreaks, requiring the further investigation on vaccine efficiency and how these viruses can overcome vaccination and cause severe lymphoid tissue damage. The second major objective of this thesis was to examine the immune response of chickens' spleens to virulent NDV-GVII through identifying candidate genes and novel pathways that may be associated with lymphocyte destruction and the molecular base for pathogenesis. The final aim of this dissertation has attempted to identify whether there are areas of the viral genome other than the fusion protein cleavage site that may contribute to an increased level of NDV-GVII pathogenicity. The main findings from each of these studies will be discussed, along with an overall synthesis and suggestions for future studies.

5.2 Key finding summary

5.2.1 Complete genome sequences of virulent NDV-GVII strains

This dissertation firstly aimed to report the complete genome sequences of two newly emerged Newcastle disease viruses, Mega/001WJ/2013 and Cimanglid/002WJ/2015 that were isolated from brains of infected birds in ND outbreaks in Indonesia in 2013 and 2015. This work was investigated and characterised in Chapter 2 where more details of viral pathogenicity, evolutionary and phylogenetic tree were addressed. The genome sequences for Mega/001WJ and Cimanglid/002WJ were deposited in Genbank with accession numbers MN688613 and MN688614 respectively. The findings revealed that two strains currently causing outbreaks in vaccinated possessed the amino acid sequences of highly pathogenic NDVs at the C terminus of the F protein cleavage site, particularly the amino acid sequence motif ^{112}RRQKRF^{117} for the Mega strain and ^{112}RRRKRF^{117} for Cimanglid strain. Additionally, the pathogenicity of both NDV isolates Mega and Cimanglid, was examined using standard protocols: the MDT assay indicated that Mega and Cimanglid are velogenic NDVs with MDT of 40h and 60h, respectively. Using the maximum likelihood method, a phylogenetic tree was constructed based on the full-length of the F gene sequence of Mega, Cimanglid and 37 previously published NDV genome sequences; Mega and Cimanglid were grouped as genotype VII.2, phylogenetically close to NDV isolates from Indonesia, China, Vietnam and Korea. Importantly, nucleotide sequence analysis showed that the amino acid identities of NP, P, M, F, HN and L proteins between current viral strains and the La Sota vaccine strain (commonly used in Indonesia) are 92%, 81%, 88%, 89%, 85% and 94%, respectively. Interestingly, the lentogenic vaccine La Sota strain has been shown to provide 100% protection against mortality after challenge with virulent NDV strains if there is good vaccination practice and enough time between vaccination and exposure to wild virus. However, the vaccine does not prevent viral shedding. One study revealed that the La Sota

vaccine can confer protection against disease caused by other virulent NDVs but does not fully protect chickens against NDV-GVII isolates (Roohani 2015). Our findings of this thesis suggest an urgent need for a newly updated vaccine strategy against increasing ND outbreaks that are caused by virulent NDV-GVII isolates worldwide.

5.2.2 Transcriptome profiling of chicken spleens after challenge with virulent NDV-GVII

The major objective of this study was to identify candidate genes and novel pathways associated with lymphocyte depletion and lymphotropic pathology of NDV-GVII strains. The transcriptomic analysis of spleens of infected chickens in response to highly pathogenic NDV-GVII was performed using RNA-seq and bioinformatics tools as characterised as Chapter 3. In our experimental study, clinical signs of gastrointestinal, respiratory or even neurological system were not observed in challenged birds, but the general hyperaemia and the decreased size of spleen and bursa of Fabricius were observed as uniquely notable signs in the challenged group. Furthermore, the other notable and unique post-mortem finding was the darkness of the breast muscles in all challenged birds that has not characterised in any previous research. The response of the lymphoid tissue's transcriptome to virulent NDV-GVII was necessary to understand a shift in the tissue tropism of virulent NDV virus. By using RNA-seq analysis, the large number of DEGs in response to NDV-GVII infection that were identified indicated the presence of viral genetic material that had a significant impact on the spleen transcriptome. This dissertation's results revealed a total of 6,361 DEGs in which 2,855 down-regulated and 3,506 up-regulated genes were associated with disease production. Pathway analysis of the DEGs between challenge and control groups showed that the majority of DEGs were enriched in: cellular immune responses; cellular growth, proliferation and development; cytokine signalling; cellular stress and injury; and humoral immune response categories.

The most significant and interesting pathway predicted by IPA in this research was fMLP signalling in neutrophils because of its known impacts on the inflammatory and innate immune responses as well as tissue destruction (Mortaz et al. 2018). Under normal conditions, neutrophils are the frontline cells of the innate immune system and play a critical role in host defence against invading microbial pathogens through interaction with chemo attractants and cytokines. Neutrophils express a variety of cell surface receptors such as heterotrimeric GPCR for N-formyl-Met-Leu-Phe (fMLP), Fc-receptors or innate immune receptors for the recognition of invading pathogens and an inflammatory environment. Activating these receptors triggers intracellular signal transduction pathways, resulting in the correct biological response such as migration, phagocytosis, superoxide production, actin reorganisation and transcriptional activation. Nevertheless, inappropriate functioning activation of neutrophils also contributes to tissue damage during inflammatory disease (Futosi, Fodor & Mócsai 2013). In the spleen, fMLP signalling in neutrophils was the most significantly downregulated pathway in challenged birds at an early stage of infection that could be involved in severe lymphoid tissue damage.

This study also revealed significant downregulation of PI*3K* signalling in B Lymphocytes in response to NDV-GVII infection. Activation of PI3K signalling at the early stages of infection has essential roles in viral replication, cell survival and proliferation in response to viruses such as avian influenza virus, NDV, and some other paramyxovirus (Guo, H et al. 2007; Kang et al. 2017). Additionally, Peters *at al.* stated that Sendai virus infection induced inhibition of PI3K signalling resulting in faster apoptosis infected cells (Peters, Chattopadhyay & Sen 2008). As a paramyxovirus, the inhibitory effect of PI3K signalling by NDV-GVII may also be correlated with enhanced apoptosis. Our recently published paper on the role of apoptosis and necrosis in response to virulent NDV shown that dysregulation of cell death related pathways was associated with the pathogenesis of NDV-GVII (Rabiei et al.

2021). Surprisingly, our findings on host-pathogen interaction in this thesis showed significant downregulation of all immunologically regulated pathways such as leukocyte extravasation signalling, NF-kB activation by viruses and other innate immune pathways. Dysregulation of these novel pathways possibly results in widespread immune suppression that could be one of the evolved strategies of the virus to evade the host immune response. Importantly, disease and function annotation analysis using IPA software also revealed the top 20 markedly decreased functions or diseases mainly relating to the quantity of lymphocytes and lymphoid cells. Our findings revealed the involvement of multiple cellular immune processes and cytokines in the pathogenesis of NDV-GVII that provides a better insight into the lymphotropic behaviour of virulent NDV-GVII.

More importantly, the molecular basis of pathogenesis may not be able to be discovered by pathway analysis alone, therefore, a wide range of bioinformatics and computational tools were employed in this thesis to identify closely related disease subnetworks and novel molecules through PPI networks. PPI network analysis revealed the top three gene modules associated with the immunopathological phenotype and disease progression. Biological function enrichment analysis of subnetworks indicated that almost all DEGs were significantly enriched in immune-response activating cell-surface receptor signalling, leukocyte migration, endocytosis, glutamate receptor signalling, long-term synaptic potentiation, and glial cell development, defence response to the virus and negative regulation of viral processes. PPI network analysis also identified eleven novel hub genes including EGF, LPAR5, AGT, AGTR1, RAC2, CD4, CD3D, IL7R, NPY, GRM3, and GRAP2 that may have important roles in severe lymphoid tissue destruction and host cellular immune response. Interestingly, most of these hub genes were pro-inflammatory and inflammatory inducible genes and all were located in the top significant subnetwork that suggests the noticeable role of the first gene module in the molecular pathogenesis of disease. Our study

finds involvement of key gene modules, hub genes and some functional biological pathways related to cellular immune responses, phagocytosis and inflammatory responses, and defence responses to the virus in the molecular pathogenesis of Newcastle disease.

5.2.3 Viral gene expression may have an impact on spleen response to virulent NDV infection

While the previous works in this dissertation have not readily investigated specific viral genes or protein sequences of interest to evaluate their role and effect on the pathogenicity of virulent NDV-GVII in vivo, a broader approach was used to identify the differential expression of individual viral genes during disease production. In order to evaluate whether there are other areas of the viral genome in addition to fusion protein cleavage site that may impact on tissue responses and pathogenesis of disease, viral mRNA expression during disease progression was analysed using qRT-PCR and RNA-seq techniques. It was hypothesized that differential expression of individual viral genes in transcription could be related to virulence and immunopathological tropism of virulent NDV-GVII through transcription and replication. Increased levels of mRNA abundance would imply more efficient transcription. After construction of qRT-PCR and RNA-seq methods to determine the relative abundance of individual viral transcripts, it was revealed that NP was the most abundant mRNA, followed by P and gradually reducing of expression levels for the next genes, whereas L showed the lowest level of expression. Although the V gene was not specifically measured, it was assumed that viral transcript counts of the V gene would be expressed in a similar abundance to the P protein mRNA. However, further works should be taken to investigate more details about this gene.

Several previous studies investigating viral transcription of La Sota virus in different tissues, such as Harderian gland and Trachea in vivo using RNA-seq (Deist, Melissa S et al. 2017; Deist et al. 2018) showed that although viral gene mRNAs were detected in both tissues, viral

transcript counts detected in Trachea were much higher than in Harderian gland. The detection of viral transcripts was associated with unique tissue responses to NDV infection regarding DEGs activating T cell related pathways in these tissues, but not in the lung. In our study transcriptomic profiling of virulent NDV was characterised using RNA-seq in vivo, which also revealed the massive viral transcript counts in the spleen as a targeted tissue of virulent NDV-GVII, while La Sota strain virus mainly replicates in trachea epithelial cells. As discussed in Chapter 3, most DEGs produced in response to virulent NDV-GVII were significantly enriched in the cell-mediated immune response pathways that are critically important in clearing NDV and reducing viral shedding (Kapczynski, DRA, C. L.; Miller, P. J. 2013; Marino & Hanson 1987). These suggest that differences of viral transcript counts within each tissue may have impacts on the tissue response to the challenge.

Recent works on determining viral transcriptional gradients of parainfluenza virus 2(PIV2), parainfluenza virus 3(PIV3), parainfluenza virus 5(PIV5) and Mumps virus belonging to *Paramyxoviridae* family have also demonstrated the mRNAs attenuation at certain gene boundaries (Wignall-Fleming et al. 2019). PIV2 and PIV5 show a steep decline at NP:V/P boundaries, PIV3 and PIV5 at HN:L and Mumps at V/P:M boundaries. In addition, while the mRNAs for PIV2 and PIV5 are clearly the highest, Mumps experiences the same number of transcripts at NP and V/P. Our thesis's results revealed that there was slightly steep attenuation of mRNA abundance at NP:P and HN:L boundaries for virulent NDV-GVII that was the most similar to PIV5. In contrast, when measuring viral transcripts in Trachea in response to lentogenic La Sota virus, Deist *et al.* stated that viral transcript counts seems to be higher for proteins at the 3'end of the virus genome (Deist, Melissa S et al. 2017). This difference in viral gene expression of the same virus but different genotypes may therefore have influenced pathogenicity. Furthermore, NP, P and L known as the viral replication complex play the most significant role in viral transcription that may be associated with the

gradient. Previous research also indicated that the activity of NP, P, and L directly contribute to virulence (Dortmans, J. C. et al. 2010). It was also revealed that NP and/or P proteins of virulent NDV not only trigger autophagy that benefit its replication through regulating ATF6 and PERK pathways, but also interact with host eIF4E to benefit viral mRNA translation (Cheng et al. 2016; Rabiei et al. 2021; Zhan et al. 2020). It was hence thought that these genes may also have a role in the pathogenicity of virulent NDV-GVII. To the best of our knowledge, this is the first-time transcriptional profiling of virulent NDV is reported in vivo using RNA-seq and qRT-PCR techniques. This investigation of mRNA abundance gradient has been limited by tissue types and number of viruses challenged. It would be helpful to produce transcription profiles of multiple pathotypes of NDVs and different tissue types to determine whether individual viral gene expression can be involved in pathogenicity.

5.3 Future directions

This dissertation has provided a better understanding of the underlying molecular basis of pathogenesis by virulent NDV-GVII through interaction between the host and viral pathogens as well as roles of viral proteins in viral transcription. The major objective of this study was identifying novel key genes and pathways associated with immunopathological tropism of NDV-GVII via transcriptome analysis of spleens of infected birds. Future work needs to include different time points and multiple tissue types to be able to uncover the host response to this virus. Due to the restriction of access to other velogenic strains and management of challenge experiments in BLS3 facilities, it was not practical for us to include additional virulent strains in this study. Hence, it would also be worthy for further studies to compare host responses to virulent NDV-GVII with other lentogenic and velogenic pathotypes of NDVs that will give a better insight into the molecular pathogenesis of virulent NDV. Additionally, it would be interesting to see whether different chicken lines will respond

differently to virulent NDV-GVII as they did with the lentogenic La Sota virus (Deist, Melissa S et al. 2017; Zhang, Jibin et al. 2018).

Although deep-sequencing analysis based on RNA-seq has been employed in this thesis, this approach only determined the differential gene expression of the polyadenylated mRNAs in response to NDV. There are a wide range of other types of RNA such as tRNA, rRNA, lncRNA, miRNA, siRNA, etc that may have an important role in the pathogenesis of this lymphotropic viruses. In future studies, the role of these other RNAs in the molecular pathogenesis of NDV should also be studied. In this thesis, due to the limitations of database for functional genomic analyses from chickens, some information on biological functions and pathway analyses were used from human, mouse and rat database that can be to some extent distinct from chickens. Although some functions have been confirmed in chicken -based database more confirmations of our hypotheses should be undertaken, and biologically functional databases need to be further developed to meet these needs for chickens.

Although the correlation between host response mRNAs level and viral mRNA abundance was also discussed in this study, it would be much clearer if future studies on transcription and replication analysis using high-throughput sequencing of NDVs with other family members, different pathotypes and tissue types will be compared.

5.4 Conclusion

This dissertation revealed that highly pathogenic NDV-GVII strains are the currently predominant circulating viruses in vaccinated flocks in Southeast Asia, based on full genome sequencing analysis. This result also indicates an urgent need to update NDV vaccines and vaccine development strategies to be able to control and prevent the prevalence of this virulent NDV-GVII in endemic areas.

Additionally, this study has also furthered comprehensive understanding host responses to virulent NDV-GVII. fMLP signalling in neutrophils, PI*3K* signalling in B Lymphocytes, Fcy

receptor-mediated phagocytosis in macrophages and monocytes, leukocyte extravasation signalling, NF-kB activation by viruses and EGF, LPAR5, AGT, AGTR1, RAC2, CD4, CD3D, IL7R, NPY, GRM3, and GRAP2 are novel pathways and key genes in this thesis that could have an important role in the molecular pathogenesis of NDV-GVII. PPI network analysis also revealed subnetworks closely associated with lymphotropic behaviour of virus including cellular immune -response activating cell-surface receptor signalling pathway, leukocyte migration, endocytosis, glutamate receptor signalling pathway, defence response to the virus and negative regulation of viral processes. This work sheds light on host responses to virulent NDV and provides key genes and novel pathways related to the pathogenesis of virus that may be useful in the prevention and treatment of disease by virulent NDV-GVII. Furthermore, through measuring viral transcript abundance in infected birds, NDV-GVII has a decreased transcription gradient with a slightly steep attenuation at NP:P and HN:L boundaries, implicating roles for NP, P and L in pathogenicity. Overall, this thesis has uncovered the underlying molecular mechanism of pathogenesis of experimentally infected birds with newly emerged virulent NDV-GVII.

6. REFERENCES

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