Molecular and Immunology Studies of Vaccinated Chickens with Newcastle Disease Virus Genotype II and Genotype VII

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A Thesis Submitted for the Degree of Doctor of Philosophy

in

School of Animal and Veterinary Sciences

The University of Adelaide
Australia

September 2021

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Putri Pandarangga,

September 2021

Acknowledgment

Thank you to Farhid Hemmatzadeh as my principal supervisor for choosing me as your Ph.D. student from many candidates who applied. It was not easy to complete this program, but Farhid always supported me with great patience. Thank you also to Milton McAllister for always providing input on every draft and helping me with my English. Thank you to Anne Peaston, who helped me a lot with writing. You guys are excellent supervisors! It’s been said that "if you want to find a supervisor, look for a supervisor who is always supportive, not a super-smart one (actually, all of you are super intelligent persons) because most supervisors who are very supportive are excellent supervisors in their fields." I found these qualities in all of you! Thank you, Gordon Howarth, for always ensuring that I was on track during my Ph.D. journey. Also, thank you to Corrie Brown for always did proofreading all my manuscripts.

So grateful to have an excellent bioinformatics team: Rick, Lloyd, and Kelly. Especially for Rick, thank you so much, Captain! I owe you a lot for editing my English. For labmates: Phuong, Mohammad, Sammi, thanks for the bitter and sweet journey during our Ph.D. program. Thanks also to Andrea as the keeper of the PC2 laboratory. Thank you also, Gayani, Sammi Khan, May, Bhumi, Farouq, Shadi, for becoming good labmates. For minibus Des's drivers: Len, Betty, Steve, and Charles, thank you so much for always driving me to school. Geez! So grateful to have you guys. Thanks to Leske peeps (Torben, Darren, Wayne, Lucy, Charles, Ryan, and Lora) for sharing the office!

Thanks to Amelia Noble and her family for always welcome me to their place. Hannah Ling, Mr. Charles Ling, and Mrs. Melinda Rankin made my life very easy when I first came to Australia. Thank you to FOCUS peeps for our friendship that built my faith in God. David Purton, thank you so much for always encouraging me to live in God and correcting my writing. Also, thanks to “the Harris” for the hospitality and proofreading of my complicated draft.

Special thank you to Esther Yip as my "crazy buddy" and my sister in Christ for showing me how to be humble and kind to people.

Thank you also to my family who lives in Sumba, Indonesia. I am so grateful that God put me in the Pandarangga family! I want to share the joy of this achievement to Bapa Pandarangga, Mama Rambu Lika, K Niko, K Sari, Mima, Yesa, K UB, om Piter, K Adi, K Essy, Dedi, Cathy, Salmon, Yanto, K Maya, Mitha, Dhea, Joanne, Sepri, mam Rey, Urey, and Renaya. Thank you!

Last but not least, my savior and my Lord, Jesus Christ. Only because of HIM I could go this far. Thank you, Jesus!

"I can do all this through Him, who gives me strength!"

Philippians 4:13

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Pandarangga P, P. T. K. Doan, A. E. Peaston, R. Tearle, W. Y. Low, K. Ren, N. P. I. Dharmayanti, F. Hemmatzadeh. Molecular Signatures of Chickens Administered Newcastle Disease Virus Genotype II or Genotype VII Vaccines. Under Review in Scientific Report Journal.

Pandarangga P, M. M. McAllister, A. E. Peaston, Yuen Tung Ngai. F. Hemmatzadeh. Performance Comparison of Homologous and Heterologous Newcastle Disease Virus in Vaccines and Antibody Tests. Planning to submit in Vaccine Journal.

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List of Abbreviations

AA: Amino Acid	IFIT: Interferon Induced proteins with Tetratricopeptide repeats
ACIAR: Australian Centre for International Agricultural Research	iGluR : ionotropic Glutamate Receptor
ACTB: Actin Beta	IgA / G / E / M : Immunoglobulin A / G / E / M
ACTG : Actin Gamma	IKK : Kappa B Kinase
ACVR2B : Activin A Receptor Type 2B	IL-6 : Interleukin-6
ACTA : Actin Alpha	IL-9 : Interleukin-9
ACTN : Actinin Alpha	IL-16 : Interleukin-16
ADCY : Adenylate Cyclase	ISGs: Interferon Stimulating Genes
Adj : Adjuvant	ITGB3: Integrin Subunit Beta 3
AE : Amplification Efficiency	JAK: Janus Kinases
AFDN : Afadin	JNK : c-Jun N-terminal kinases
Ag : Antigen	KCNJ6 : potassium inwardly rectifying channel, subfamily J, member 6
ANOS1: Anosmin 1	LAIV : Live Attenuated Influenza Virus
AMPAR : α amino-3-hydroxy-5 methyl-4-isoxazole Propionic Acid Receptor	Le : Leader
APCs : Antigen-Presenting Cells	LFC : Log Fold Change
AP-1: Activating Protein-1	LGP2 : Laboratory of Genetics and Physiology 2
ATF : Activating Transcription Factor	L : Large Protein
BAM : Binary Alignment Map	LTP : Long-Term Potentiation
Ban/AF : Banjarmasin/010/10/modification F protein	LTD : Long-Term Depression
BBALITVET : Balai Besar Penelitian Veteriner	MAVS : Mitochondrial Antiviral Signaling Protein
BCL 2 : B-cell lymphoma 2	Mab : Myeloid Differentiation Primary
BDNF : Brain-Derived Neurotrophic Factor	MAP : Molecule Activity Predictor
BM : Bone Marrow	MAPK : Mitogen-Activated Protein Kinase

BMX : Bone Marrow Tyrosin Kinase Gene in Chromosome X	MATK : Megakaryocyte-Associated Tyrosine Kinase
BLAST : Basic Local Alignment Search Tool	MDA5 : Melanoma Differentiation-Associated Protein 5
BSL-3: Biosafety level 3	mGluR : metabotropic Glutamate Receptor
CA-NDV : California Newcastle Disease Virus	MHC : Major Histocompatibility Complex
CACNA : Calcium Voltage-Gated Channel Subunit Alpha	MMP 1 : Matrix Metallopeptidase 1
CAMK 4 : Calcium/Calmodulin Dependent Protein Kinase IV	M : Matrix Protein
CAMP : Cathelicidin Antimicrobial Peptide	MyD88 : Response Gene 88
CARD : Caspase Recruitment Domain	MYL : Myosin Light Chain
Caspase-1 : Cysteine Aspartic Protease 1	NDV : Newcastle Disease Virus
CCR7 : C-C Motif Chemokine Receptor 7	NEMO : NF-Ƙb Essential Modulator
CCL5 : C-C Motif Chemokine Ligand 5	NFAT : Nuclear Factor of Activated T-Cells
CDH 1: Cadherin 1	NF-κB : Nuclear factor kappa B
cDNA : complementary DNA	NFKBIE : NFKB Inhibitor Epsilon
CD 4+ : Cluster of Differentiation 4 +	NHMRC : National Health and Medical Research Council
Cdc42 : Cell Division Cycle 42	nm : Nano Meter
CEBPB : CCAAT Enhancer Binding Protein Beta	NLGN 1 : Neuroligin 1
CeA : Central Nucleus of the Amygdala	NOX3 : NADPH Oxidase 3
chIFN-α : Chicken Interferon Alpha	NTF3 : Neurotrophin 3
chIFN-β : Chicken Interferon Beta	nt : Nucleotide
chiTLR : Chicken Toll Like Receptor	NRXN1 : Neurexin 1
CLDN : Claudin	OASL : 2'-5'-Oligoadenylate Synthetase Like
CMI : Cell-Mediated Immune	OIE : Office International des Epizooties
CNTNAP2 : Contactin Associated Protein 2	OASL : 2'-5'-Oligoadenylate Synthetase Like
CNS : Central Nervous System	PAMPs : Pathogen-Associated Molecular Patterns

CPM : Counts Per Million
CREB : cAMP response element-binding protein
CRP : C-Reactive Protein
CSK : C-Terminal Src Kinase
Ct Cycle : Threshold
CXCR4 : C-X-C Chemokine Receptor Type 4
CXCL13 : C-X-C Motif Chemokine Ligand 13
CX3CR1 : C-X3-C Motif Chemokine Receptor 1
CYBB : cytochrome b-245
DCs: Dendritic Cells
DEGs : Differentially Expressed Genes
DNA: Deoxyribonucleic Acid
DLC1 : Deleted in Liver Cancer 1
DNA : Deoxyribonucleic Acid
DEG : Differentially Expressed Genes
dsRNA : Double-Strand RNA
EGFR : Epidermal Growth Factor Receptor
EGR 1 : Early Growth Response 1
ELISA : Enzyme-Linked Immunosorbent Assay
EFNA5 : Ephrin A5
EID_{50} : Embryo Infection Dose50
EIF2α : Eukaryotic Translation Initiation Factor 2A
EPH : Ephrin Type-A Receptor 1

PBS : Phosphate-buffered saline
PERK : PKR-like endoplasmic reticulum kinase
PIK3C2G : Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2 Gamma
PKCθ : Protein Kinase C Theta
PLA 2 : Phospholipase A2 Group IIA
PLEKHA2 : Pleckstrin Homology Domain Containing A2
PPMV-1 : Pigeon Paramyxovirus-I
P2RX7 : Purinergic Receptor P2X 7
PP-1 : Protein Phosphatase-1
P : Polymerase-Associated Protein
PRKAR2B : Protein Kinase CAMP-Dependent Type II Regulatory Subunit Beta
PRRs : Pattern Recognition Receptors
PRKCH : protein kinase C eta
PTK2B : Protein Tyrosine Kinase 2 Beta
PTPRC : Protein Tyrosine Phosphatase Receptor Type C
PVN : Paraventricular Nucleus
PTPRC : Protein Tyrosine Phosphatase Receptor Type C
PRKAR2B : Protein Kinase CAMP-Dependent Type II Regulatory Subunit Beta
qPCR : quantitative PCR
RAC 2 : Rac Family Small GTPase 2
RET : Ret Proto-Oncogene
RIG-I : Retinoic Acid-Inducible Gene I
ROR2 : Receptor Tyrosine Kinase Like Orphan Receptor 2

ERBB4 : Erb-B2 Receptor Tyrosine Kinase 4
ER : Endoplasmic Reticulum
ERK : Extracellular Signal-Regulated Kinases
EYA4 : Eyes Absent 4
FAO : Food and Agriculture Organization
FADD : Fas-Associated Death Domain Protein
F : Fusion Protein
FDC : Follicular Dendritic Cells
FDR : False Discovery Rate
GAPDH : Glyceraldehyde 3-Phosphate Dehydrogenase
GABRA : gamma-aminobutyric acid (GABA) receptor
GABRB : Gamma-Aminobutyric Acid Type A Receptor Subunit Beta1
GABBR2 : Gamma-Aminobutyric Acid Type B Receptor Subunit 2
GABRG : Gated Gamma Aminobutyric Acid Receptor
GAB2 : GRB2 Associated Binding Protein 2
GalGal6 : Gallus-gallus
GC : Germinal Center
GNAS : Guanine Nucleotide Binding Protein, Alpha Stimulating
GRIA1 : Glutamate Ionotropic Receptor AMPA Type Subunit 1
GRIN : Glutamate Ionotropic Receptor NMDA Type Subunits
GRAP2 : GRB2 Related Adaptor Protein 2
GRM 1: Glutamate Metabotropic Receptor 1
GRB2 : Growth Factor receptor Bound Protein 2
RLRs : RIG-I Like Receptors
RNA-seq : RNA Sequencing
RNP : Ribonucleoprotein
RNA : Ribonucleic Acid
RT – PCR : Reverse Transcription PCR
RBC : Red Blood Cell
RIP : Receptor-Interacting Protein
SLCO1C1: Solute Carrier Organic Anion Transporter Family Member 1c1
SNC : synuclein
SOCS2 : Suppressor Of Cytokine Signaling 2
SPF : Specific-Pathogen-Free
SNCA : Synuclein Alpha
ssRNA : Single-Strand RNA
STING : Stimulator Of Interferon Genes
SYT : Synaptotagmin
STXBP6 : Syntaxin Binding Protein 6
STINTBAB : Similar to NAP1 TBK1 Adaptor
STAT : Signal Transducer And Activator Of Transcription
STYK1 : Serine/Threonine/Tyrosine Kinase 1
TLR : Toll Like Receptor
TMM : Trimmed Mean of M-values
TNF Tumor Necrosis Factor
TGFβ : Tumor Growth Factor Beta

GVIIvacc : Genotype VII Vaccine
GIIvacc : Genotype II Vaccine
GTPase : Guanosine Triphosphatease
HA : Haemagglutination Test
HAU : HA Unit
HI : Hemagglutination Inhibition
H5N1 : Hemagglutinin 5 and neuraminidase 1
HLA : Human Leukocyte Antigen
HLA-IL1R1 : Human Leukocyte Antigen-Interleukin 1 Receptor Type 1
HN : Haemagglutination-Neuraminidase
HR-A : Hepta Repeat A
HSP : Heat Shock Protein
ICTV : International Committee On Taxonomy Of Viruses
iCOS : Inducible T-Cell Costimulator
iCOSL : Inducible T Cell Costimulator Ligand
IFN : Interferon
IFNAR : Interferon Alpha and Beta Receptor
IFNGR : Interferon Gamma Receptor
ILK : Integrin Linked Kinase
IL1R2 : Interleukin 1 Receptor, Type II
iNOS: Inducible Nitric Oxide Synthase
IPA: Ingenuity Pathway Analysis
IRE: Iron-Responsive Element
IRF: Interferon Regulatory Factors
IRAKs: Interleukin-1 Receptor-Associated Kinase
IKKγ Iƙb Kinase Gamma

TRIF : TIR-Domain-Containing Adapter-Inducing Interferon-Β
Tr : Trailer
Tfh : T Follicular Helpers
Th1 : T Helper 1 Cells
THBS2 : Thrombospondin 2
TAB1 : TAK1 Binding Protein 1
TIMP4 : Tissue Inhibitor Of Metalloproteinase-4 Gene
XBP-1 : X-Box Binding Protein 1
XCL : X-C Motif Chemokine Ligand 1
YF-17D : Yellow Fever-17d
ZAP70 : Zeta Chain Of T Cell Receptor Associated Protein Kinase 70

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Abstract

Since it was discovered in 1926 in England and Indonesia, Newcastle Disease Virus (NDV), especially from Genotype VII (GVII), has caused death in chickens that have been vaccinated using the NDV vaccine Genotype II (GII), known as a heterologous vaccine. Vaccines from the GVII strain, also known as homologous vaccines, have prevented NDV outbreaks. Determination of the differences between the two vaccines was done using a transcriptomic method to determine the response of chickens at the genetic level; with serology approach and viral loading counting; and differences between the two strains genetically using whole-genome sequences. Thirty, three-week-old specific-pathogen-free (SPF) chickens were divided into three groups. The first group was a negative control, the second group was vaccinated with GIIvacc, and the third group was vaccinated with GVIIvacc. Treatment groups were immunized with vaccines on day 14 and day 28. Sera were obtained from all groups on day 28 for the serology tests. On day 42, the spleen was collected for transcriptomic. Meanwhile, the whole genome sequence samples were obtained from the NDV outbreak in 2015 in Indonesia, Genotype II strain, and challenge strain to determine vaccine effectiveness

Spleen transcriptomic showed that GVIIvacc down-regulates the neuroinflammation pathway but increases the communication activity among neurons as part of the synaptogenesis pathway. Thus, it is speculated that suppressing the neuroinflammation pathway is associated with protecting the nervous system in chickens from excess leukocytes and cytokine activity. Meanwhile, GIIvacc only prevents apoptosis by suppressing PERK/ ATF4/CHOP as part of the unfolded protein response (UPR) pathway. Thus, the use of GVIIvacc should be considered in countries where GVII strain causes NDV outbreaks.

The transcriptomic result aligned with serological and challenged virus test that homologous vaccine (GVIIvacc) gave better protection by reducing the viral shedding and had higher protective antibodies than a heterologous vaccine (GIIvacc). In particular, the Hemagglutination Inhibition (HI) test showed that antibody titers were higher when tested with homologous antigen. However, the cleavage site of the Fusion (F) protein from GII and GVII were used as alternative antigens in an ELISA, did not perform well to obtain the relevant antibody titer. After being challenged with GVII, viral shedding from vaccinated chickens with GVIIvacc was significantly reduced compared to chickens vaccinated with GIIvacc. Both chicken groups showed no clinical signs.

The whole-genome sequence and phylogenetic tree results showed that GVII is still the dominant NDV strain that causes NDV outbreaks in Indonesia. In addition, ITA strains for testing the vaccine's efficacy belong to GVI. Hence, using GVI as a heterologous strain from the field as a challenge strain for effective vaccine testing should be considered in veterinary laboratories, especially in Indonesia. All the results from my research study suggested that to combat NDV, the vaccine, antigen for antibody titer, and antigen as challenge strain for effectivity vaccine need to be homologous or coming from the NDV genotype, which causes an outbreak in the field.

Thesis Outline

This thesis contains five chapters:

Chapter 1 is a general introduction and literature review. This chapter is about Newcastle Disease Virus (NDV) characteristics, vaccine development, and how NDV vaccines produced worldwide affect the innate and adaptive immunity in chickens at the molecular level and economic impact caused by this virus. This chapter will be submitted for publication. Additionally, the type of methods used to answer research questions in this research program was explained in this chapter.

Chapter 2 predicts the gene expression differences in vaccinated chickens with NDV vaccine genotype II and genotype VII by applying spleen transcriptomic. This chapter is written in publication format and has been submitted in the Scientific Report Journal.

Chapter 3 compares homologous and heterologous NDV in vaccine and antibody tests to predict the relevant protective antibodies. Similar to the previous chapter, this chapter is also written in publication format and submitted in the Vaccine Journal.

Chapter 4 is whole-genome sequencing from NDV isolates that caused outbreaks in Indonesia. Genotype VII is still responsible for the NDV outbreaks in Indonesia. This chapter has been published in Microbiology Resources Announcements Journal.

Chapter 5 is a general discussion that contains a summary and conclusion of the whole thesis.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. 1. General Introduction

Newcastle Disease Virus (NDV) is the cause of Newcastle Disease (ND) in birds. The number of bird species infected by this virus is 236 [1]. NDV has generally been categorized into two classes, and virulent NDV comes from class II [2]. The dominant genotype causing outbreaks worldwide belongs to genotype VII (GVII) [3-5]. Since this disease was reported for the first time, genotype I (GI) or genotype II (GII) have been used as commercial vaccines worldwide, including in Indonesia [6]. Xiao (2012) has proven that there is an antigenic difference between GVII and GII [7]. The difference is one of the important contributing factors in NDV outbreaks where commercial vaccines cannot protect chickens. Regardless of the pros and cons, homologous vaccines or NDV vaccines from the field are considered as a solution in preventing NDV outbreaks [8,9].

The difference between homologous vaccines and commercial vaccines in serology tests and viral shedding load after the exposure to the virulent NDVs is still debated among researchers focusing on NDV [9,10]. However, the difference in the response of chickens to these two vaccines at the molecular level is still obscure. Furthermore, the mechanisms of the vaccines in immune responses are complex, so vaccine studies in humans have applied a system biology approach to finding out which genes interact with each other to build networks [11]. Thus, this approach provides ample evidence about how the vaccine or antigen stimulates the immune system than using only a few components of vaccine responses [11,12]. Therefore, by using spleens transcriptomics in our study, which is a part of the system biology approach, we hoped to learn the molecular differences and network pathways of how NDV vaccines stimulate chickens' immune response or other responses that support chicken protection.

Moreover, this study performed serological tests, haemagglutination inhibition (HI) and enzyme-linked immunoassay (ELISA), to investigate whether homologous vaccines derived from GVII NDV induce higher antibody titers than commercial vaccines. Specifically, chicken sera were tested with whole virus antigens, including GI, GII, and GVII using an HI test, and the peptide of the cleavage site of F protein from GII and GVII using an ELISA. These tests aim to determine the antibody titers from homologous vaccines that could give relevant protective antibody titers useful in NDV surveillance. Then, when challenged by using the GVII NDV, whether the homologous vaccine can reduce viral shedding compared to the GII vaccine (heterologous vaccine) will also be investigated in this study. Finally, by using the full-genome sequencing method, two isolates that cause outbreaks in Indonesia needed to be characterized, and a phylogenic tree was constructed.

Hence the result of this study is to know whether vaccines, antigens, and viral challenges that are homologous to NDV in the field can be recommended in places where GVII NDV is prevalent. Moreover, another purpose of this study is to know whether the antigen strain and viral challenge strain need to be similar to field strains for predictive vaccine efficacy. In addition, the results of molecular signatures in significant pathways and genes can be used as basic knowledge in developing vaccines that can directly trigger genes that regulate the immune system in chickens.

1. 2. Literature Review

1. 2. 1. Characteristic of NDV

NDV is a single-strand RNA virus that causes Newcastle Disease. Based on the new nomenclature from the International Committee on Taxonomy of Viruses (ICTV) agreement, NDV belongs to the genera *Orthoavulavirus* in the *Paramyxoviridae* family [13]. NDV has negative single-strand and non-segmented RNA with length 15,186, 15,192, or 15,198 [13,14]. The structure of NDV genomes is 3’-Le-NP-P-M-F-HN-L-Tr-5’ where the leader (Le) and trailer (Tr) as extracistronic sequences [15,16]. In general, the length of each NDV genome is a variety where NP gene: 1747 nucleotide (nt), P gene: 1451 nt, M gene: 1241 nt, F gene: 1792 nt, HN gene: 2002 nt, and L gene: 6703 nt [14]. NDV genes encode proteins with the same name as nucleocapsid protein (NP), polymerase-associated protein (P), matrix protein (M), fusion protein (F), haemagglutination-neuraminidase (HN), and large protein (L) [15]. The P gene can encode P protein and V protein by inserting a single Guanine (G) residue in messenger RNA (mRNA) editing [17].

Generally, the shape of NDV is pleomorphic but tends to be a round figure [18]. In the center of the NDV, there is a ribonucleoprotein (RNP) [18]. N-RNA complex is genome RNA encapsulated by nucleoprotein, becoming ring- and herringbone-like particles [10] with 17 nm in length and 5 nm in the central canal [18,19]. RNP is surrounded by matrix (M) protein [13]. Moreover, M protein is covered by two phospholipid layers that distribute over the NDV membrane [20].

In NDV, two glycoprotein spikes on the surface of the virus with the head, neck, and stalk known as HN and F protein can attach and fuse, respectively, on the membrane of host cells [13,18,20]. The primary function of HN protein is to recognize and bind to the receptors containing sialic acid on the surface of red blood cells [18,21,22]. Besides that, HN protein is essential for inducing neutralizing antibodies in the host [23]. Also, the neuraminidase activity of the HN protein can diminish sialic acid from the surface of the virus to prevent viral self-agglutination and contribute to the fusion activity of F protein to allow entry of the virus into the host cell [24]. The F0 protein is cleaved by a trypsin or protease enzyme at the cleavage site to become heterodimer F1 and F2, linked by a disulfide bond [22].

The virulence of NDV is determined by F, HN, and V proteins. The amino acid motif at the cleavage site of F protein is part of the virulence determinant of NDV [25-27]. F0 in low virulent NDVs will only be cleaved extracellularly by trypsin produced in the respiratory and intestinal tract. In contrast, F0 in virulent NDV is cleaved intracellularly by proteases present in most host cells [22,26]. Besides the cleavage site of the F protein, the stem region and head of HN protein are responsible for determining the virulence of NDV [25]. Moreover, the V protein has a role in NDV virulence and virus replication [28]. Also, this protein inhibits alpha-interferon (IFN) antiviral activity by degrading the Signal Transducer and Activator of Transcription 1 (STAT1) protein of the host's innate immune response [29].

1. 2. 2. Significant Impacts of NDV

Since NDV was reported for the first time in England and Indonesia in 1926, it has been responsible for five panzootic [30]. Based on the F gene-coding region, NDV is divided into two classes. Class I only has one genotype where, previously, it was split into nine genotypes based on the partial nucleotide sequences of the F gene [31,32]. Class II is divided into 21 genotypes: I-XXI [31,33-37]. The first panzootic involved genotypes II, III, and IV, which started in Indonesia and England between 1920 to 1960 [38,39]. The second panzootic spread from Europe to the USA, four years after the first panzootic [22,40,41]. Genotype VI had a significant role in this panzootic [42]. A third panzootic originated in the Middle East in the 1980s, where Pigeon Paramyxovirus-I (PPMV-1) with sub-genotype VIb was responsible [22,43]. Unvaccinated racing pigeons from the Middle East to Southeast Asia, South America, and Western Europe contributed to a fourth panzootic [37,40,44]. This panzootic was caused by Genotype VII [39]. Sub-genotype VIIi and VIIh, XIIIa drive recent global outbreaks, and XIII are suggested to generate the current fifth panzootic [30,39].

NDV causes economic loss worldwide. For example, when the NDV outbreak occurred in 1998-2000, it impacted the economic stability of the chicken industry in Australia [45,46]. Also, NDV outbreaks have significantly impacted the poultry industry in developing countries where chickens and eggs are the primary protein sources, particularly in Indonesia [47]. The NDV outbreak reported in 2009 and 2010 in Indonesia caused 70-80% mortality in vaccinated chickens with a commercial vaccine, LaSota vaccine, which belongs to GII [48]. In addition, the economic losses caused by the NDV outbreaks in America in 2002-2003 amounted to $ 200 million [49].

1. 2. 3. Type of ND Vaccines

Since 1948, the first vaccine was developed from virulent NDV Roakin-strain and, within two years, the B1 and LaSota strain vaccines derived from GII were developed [50]. Until now, the LaSota vaccine derived from GII is a commercial vaccine that has been used worldwide. One example of a country that uses this vaccine is Indonesia, where 60% of NDV vaccines contain this viral strain [6]. Another genotype (GI) of NDV from class II used as live vaccines is chicken/Australia/QV4/1966 and chicken/Ireland/Ulster/1967 isolates [32,51].

A study reported antigenic differences between commercial vaccines and circulating NDV, namely CA-NDV, causing outbreaks in the USA [49]. In addition, at the end of 1999, it was found that GVII was the other cause of NDV outbreaks. Genotype VII has been circulated globally in the Middle East [52,53], Asia including Indonesia [32,37,39,54], South America [44], and South Africa [55]. A vaccine derived from NDV that is spreading in the field is known as a homologous vaccine [9].

The ND vaccines that have been developed worldwide consist of live attenuated, inactivated, vectored, and antigenically matched engineered NDV vaccines, summarized in Table 1.1. [56,57]. Each vaccine has advantages and disadvantages. Despite the possibility of reversion to virulence and cold-chain requirements, live attenuated vaccines can activate humoral and cell-mediated immune (CMI) responses in chicken and are less expensive [57]. In comparison, inactivated vaccines can induce a humoral antibody response, but this takes more time than live vaccines [56]. Also, the price of inactivated vaccines is higher. Based on Dimitrov's review, the vector NDV vaccine gives varying results in stimulating the immune system and is not yet available in the market [58,59]. The advantage of antigenically matched vaccines is the possibility of reducing viral shedding when challenged with a homologous virus; still, because NDV is an RNA virus that quickly mutates, the vaccine may not remain homologous [56,60].

Table 1.1. Type of ND Vaccines that Have Been Developed Worldwide

Group Of NDV Vaccines	**Vaccine Name**	**Challenge Strain**	**Immune System Response of Chicken**
Live Attenuated Vaccine (2008)	Vilegas-Glisson/University of Georgia (VG/GA) [61]	Velogenic Texas GB NDV strain	Inducing IgA in the upper respiratory tract, bile, and intestine; Providing 95-100% protection after being challenged with velogenic NDV Texas GB strain
Live Attenuated Vaccine (2012)	Reverse genetic of NDV strain Banjarmasin/010/10 (Ban/AF) [62]	NDV strain Banjarmasin/010	Increasing the neutralization and HI antibody; Preventing mortality and reducing the viral shedding after being challenged with NDV strain Banjarmasin (GVII)
Live Attenuated Vaccine- As Antigenic-	Reverse genetic of GVII isolate SG10 (aSG10) [63]	SG10 strain (GVIId)	Preventing chicken death and reducing the viral shedding after being infected with NDV SG10 strain

Matched Vaccine (2015)			
Live Attenuated Vaccine- As Antigenic-Matched Vaccine (2017)	Virulent recombinant GM of GVII (rGM-VIIm) [64]	GM NDV strain	Preventing chicken death and reducing viral shedding after challenged with GM NDV strain
Live Attenuated Vaccine (2018)	Recombinant of NDV strain R2B (rNDV-R2B) [65]	Virulent R2B NDV strain	Stimulating proliferation of CD4+ and CD8+ in chicken; Reducing viral shedding after being infected with virulent R2B NDV strain
Live Attenuated Vaccine- As Antigenic-Matched Vaccine (2018)	Reverse genetic of NDV GVII as a mutant (G7M) [66]	Genotype VII NDV (G7 strain)	Elevating homologous antibody, CD8+ T cell percentage, T cell proliferation, and IFN-γ; Reducing the challenge virus replication and shedding in chicken
Live Attenuated Vaccine- As	rLS-XIIm [67]	Genotype XII (PP2011)	Providing full protection; Decreasing viral shedding

Antigenic-Matched Vaccine (2019)			after being challenged with homolog strain.
Live Attenuated Vaccine-As Antigenic-Matched Vaccine (2020)	mIBS025 [68]	IBS002/11	Increasing antibody; Protecting from mortality and morbidity; Reducing viral shedding.
Live Vaccine (2020)	NDV isolate APMV-1/chicken/China/PT3/2016vaccine (PT3) [69]	NDV SD strain (GVIId), NDV DY strain (GVIb).	Protecting chicken from death and reducing viral shedding after being infected by GVIId and GVIb
Inactivated Vaccine (2011)	Reverse genetic NDV strain JS5/05 with F protein from non-virulent NDV (NDV/A14) [9]	NDV JS3/05 strain (GVII)	Inducing antibody; Preventing mortality and reducing viral shedding after being challenged with GVII NDV strain JS3/05
Inactivated Vaccine- As Antigenic-Matched Vaccine (2017)	NDV O/A14 [70]	NDV strain JS-14-12-Ch	Reducing viral shedding after being challenged with NDV strain JS-14-12-Ch

NDV-Vectored Vaccine (2020)	Recombinant NDV vector from LaSota with VP2 as part of IBDV (rLaSota/VP2) [58]	NDV strain Texas GB and IBDV variant GLS-5	Inducing antibody response against both NDV and IBDV; 90% protective against NDV strain Texas GB and IBDV strain GLS-5
NDV-Vectored Vaccine (2013)	Reverse genetic of NDV LaSota that expressing the glycoprotein (G) of avian Metapneumovirus subtype A or B (rLS/aMPV-A G or rLS/aMPV-B G) [59]	Velogenic NDV CA02 strain and pathogenic aMPV	Both vaccines protected chicken death from NDV strain CA02; Both vaccines only partially protect the chicken from aMPV strain
NDV-Vectored Vaccine (2014)	Recombinant NDV (rNDV) with gD part of Infectious Laryngotracheitis Virus (ILTV) - rNDVgD [71]	NDV Texas-GB and ILT	Inducing higher neutralizing antibodies; Elicit immune response for both NDV and ILTV

1. 2. 4. Chicken Innate Immunity Against NDV at Molecular Level

Like the other developed animals, innate immune response and adaptive immunity play roles in immunity to viral pathogens in chicken [72]. However, there are several different nomenclatures between mammals and birds, namely neutrophils are referred to as heterophils, and immunoglobulin (Ig) G is known as Ig Y. Innate immunity is activated when receptors, known as pattern recognition receptors (PRRs), are introduced to pathogen-associated molecular patterns (PAMPs). These pathogens consist of parasites, bacteria, and viruses. However, this study is emphasized that how the chicken immune response to the ssRNA virus, specifically NDV, at the molecular level. PRRs contain four receptor families: toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) where each receptor in the host cell can sense specific antigenic ligands [73-76]. PRRs are located either on the host cell surface or within the cytoplasm, such as phagosome, endolysosome, and endoplasmic reticulum [75,77].

NDV is an ssRNA virus as a part of PAMPs, which is sensed by specific receptors in chicken leucocytes, inducing early or innate immunity in response to viral RNA [72,73,78]. Like other animals, chickens also have TLRs, known as chicken TLRs (chTLRs). This receptor contains chTLR2, chTLR3, chTLR4, chTLR5, chTLR7, chTLR8, chTLR15, chTLR21, and two chTLR1/6/10 orthologs [79-84]. However, only chTLR3, chTLR7, chTLR15 can sense ssRNA of NDV [85-88]. Moreover, melanoma differentiation-associated protein 5 (MDA-5) and RIG-I as part of RLR located in the cytoplasm can recognize NDV [86,89]. These receptors are located on the cell surface (chTLR15) and inside the cell (chTLR3, chTLR7, MDA-5, and RIG-I). Immune response activation in NDV-infected chickens varies greatly depending on the degree of virulence in chickens where virulent NDV elicits strong immunity [85,87,90].

Binding between PAMPs and PRRs activates downstream protein adaptors to stimulate transcription factors in inducing an immune response in chickens (Figure 1.1). The type of protein adaptors for RNA viral infection are myeloid differentiation primary response gene 88 (MyD88), toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF), and mitochondrial antiviral signaling protein (MAVS) [91]. Every protein adaptor binds to its receptor to activate transcriptional activators such as NF-ßB, AP-1, IRF3, and IRF7, inducing the pro-inflammatory cytokine and interferon involved in antiviral immunity [91].

Specifically, based on the protein adaptor, the TLR signaling pathway is divided into the MyD88- and TRIF-dependent pathway [75]. However, MAVS-dependent signaling pathways rely on RLR, specifically RIG-I and MDA-5, to activate downstream molecules for antiviral response [91].

The TRIF-dependent signaling pathway involves TLR3 as receptors to activate type I IFN and interferon-stimulated genes (ISGs) via IRF3 (Figure 1. 1A) [91]. chTLR3 is a receptor capable of detecting NDV from LaSota and Hert strains [85]. As in mammals, TLR3 located in the endolysosome binds to the adaptor protein TRIF to activate transcription factors such as IRF3, NF-κB, and AP-1, stimulating pro-inflammatory cytokine, type I IFN, and ISGs [92-94]. The specific downstream molecule activated by TRIF after being stimulated by NDV is not known yet. However, by using an RNA virus model such as influenza H5N1, downstream molecules recruited by TRIF are TRAF3 and TRAF6 with the help of ubiquitin-specific protease 25 (USP25), inducing TRAF3 member-associated NF-B activator (TANK)-binding kinase1 (TBK1) and IKKε [95,96]. In general, chicken TBK1 has a vital role in regulating IRF3, IFNβ, and IRF7 to stimulate type I IFN and ISGs in response to viral infection [94]. While TRAF 6 stimulates NF-ƙB via activation of some complex such as RIP1, tumor necrosis factor receptors (TNFR)-associated death domain protein (TRADD), and Fas-associated death domain protein (FADD) [91]. Subsequently, NDV was inhibited viral replication by type I IFN and pro-inflammatory through TLR3 activation [85].

TLR7 activates the myD88-dependent pathway (Figure 1. 1B) and TLR15 (Figure 1. 1C) that are upregulated when infected with virulent NDV, activating transcriptional factor NF-κB [97,98]. There have been no studies investigating the downstream molecules stimulated by NDV, specifically. However, in general, the binding between antigen and TLR induces the IL-1R-associated kinase (IRAK) family, which comprises IRAK1, IRAK2, and IRAK4 [91,99]. IRAK-1 and IRAK-2 are activated downstream by IRAK-4 [100]. IRAK1 is phosphorylated and released from MyD88 to interact with and activate tumor necrosis factor (TNF) R-associated factor 6 (TRAF6), which then stimulates IκB kinase (IKK) [76,101,102]. In IκB kinase (IKK) activation, TRAF 6 involves two intermediate factors: a) Ubc13 and Uev1A to form Lys 63 (K63)-linked polyubiquitin chain to mediate IKK activation, b) TAK1 and TAK1-binding proteins (TAB1 and TAB2) to phosphorylate inhibitor of kappa B kinase (IKKβ) and MKK6 [103,104]. Moreover, the activation of MKK6 stimulates MAPK, including JNK1/2 and ERK1/2, to translocate and activate AP-1 in the nucleus. On the other hand, the IKK complex that contains IKKα, IKK and NF- essential modulator (NEMO) phosphorylated IKBαstimulate its degradation and trigger the translocated process of NF-κβ into the nucleus. NF-ƙB and AP-1 are transcriptional factors that induce the expression of pro-inflammatory and cytokine genes [105].

In addition, receptors in the cytoplasm capable of recognizing NDV are MDA-5 and RIG-I, where these two receptors interact with MAVs adapter proteins in activating IRF3/7 (Figure 1. 1D) [86,89]. RIG-I and MDA-5 consist of two N-terminal caspase recruitment domains (CARD) that bind to K63-polyubiquitin chains essential for IRF3 activation [106]. Thus, K63-polyubiquitin chains play a significant role in stimulating MAVS located in mitochondria [107]. Furthermore, MAVS can activate IRF3/7 and NF-ƙB via two different pathways involving TRAF3 and TRADD, respectively [108]. Specifically, IRF3/7 activation through eyes absent 4 (EYA4)/TRAF3/NF-κB activating kinase-associated protein 1 (NAP1)/similar to NAP1 TBK1 adaptor (STINTBAD) complex has a role in starting type I IFN while activation of NF-ƙB through TRADD/FADD/Caspase-8/-10 signaling cascade to stimulate pro-inflammatory cytokines [91]. Then IRF7 induces type I IFN, which affects NDV replication [109].

Moreover, chickens infected with virulent NDV, especially genotype VII, induce type I IFN and type II IFN [97,110] and chemokine [87]. Interferon response to NDV infection varies depending on the type of NDV and duration of infection involving type I and II IFN [111]. IFN-γ as part of type II IFN, produced by Th1 lymphocytes, plays a vital role in controlling the immune responses to NDV infections and reducing the pathogenicity of virulent NDV in chickens [111,112]. In addition, IFN-λ responds to viral infections by stimulating inducible nitric oxide synthase (iNOS) from macrophages and protecting the chicken embryo from viral infections [113,114].

Each transcriptional factor has a unique ability to activate cytokines. Turner, in his review, divided cytokines based on the immune response: a) pro-inflammatory consists of IL-1, IL-6, TNFα, IL-17, type 1 IFN, type II IFN, and type III IFN; b) anti-inflammatory cytokines consist of IL-12 and IL-10 [115]. Pro-inflammatory cytokines mainly were activated by transcriptional factor NF-ƙB and AP-1 via MAPK [105,115]. Meanwhile, transcription factors IRF3 and IRF7 could stimulate type I IFN as part of the pro-inflammatory cytokine response [116,117]. In addition, IL-6 has a role in the differentiation process of B-cells into plasma cells that produce antibodies [118]. Also, IL-6 binds to its receptor, IL-6R, on surface cells that activate Janus kinases (JAKs)-STAT signaling [119], especially for up-regulation of interferon stimulating genes (ISGs) ISG15, ISG20, OAS1, OAS2, and MX2 in rabies infection [120].

Interferon is also part of the pro-inflammatory cytokine response; it is an antiviral activated by viral nucleic acids. IFN in avian is divided into three types, namely type I, II, and III IFN, where IFN is analogous to mammals [121,122]. IFN-α, IFN-β, IFN-κ are produced by monocyte and dendritic cells [123] as part of type I IFN. The role of type I IFN is as an antiviral by limiting the viruses spreading, can induce innate and adaptive immunity, and stimulate autophagy [124,125]. IFN-α and IFN-β bind to their receptors IFNAR1 (Figure 1. 1E) and IFNAR2, respectively, and both have different activities in activating ISGs, whereas IFN-α is more potent in activating ISGs [126,127]. Type II IFN has only one member, namely IFN-γ [121]. Type II IFN is a regulator of the immune system and a bridge between innate and adaptive pathways [128]. In chickens, IFN-γ has receptors, namely IFNGR1 and IFNGR2 [129,130]. Type III IFN in humans is IFN-λ which consists of IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4 [131,132] but only IFN-λ3 which has similarities with chicken IFN-λ (chIFN-λ) [114]. Type III IFN is a key player in mucosal epithelial immunity by limiting viral infection [131]. chIFN-λ has a receptor, chIL28RA [133].

The types of ISGs that are activated by virulent NDV (NDV-CA02) are mature avidin, IFIT-5, Mx protein, PKR, SOCS-1, SOCS-3, and 12, 2'-5'-Oligoadenylate Synthase (OAS) (Figure 1. 1E) [87]. Another ISG is cViperin that suppresses NDV replication by interacting with the matrix protein in NDV [134]. Mx can inhibit the NDV replication [135]. The types of pro-inflammatory cytokines activated by NDV infection vary greatly depending on the level of virulence. One example of NDV virulence is NDV-CA02 and up-regulated pro-inflammatory genes, i.e., IL-6 and IL-1β [87]. Still, in the same study, the LaSota strain did not significantly stimulate these genes.

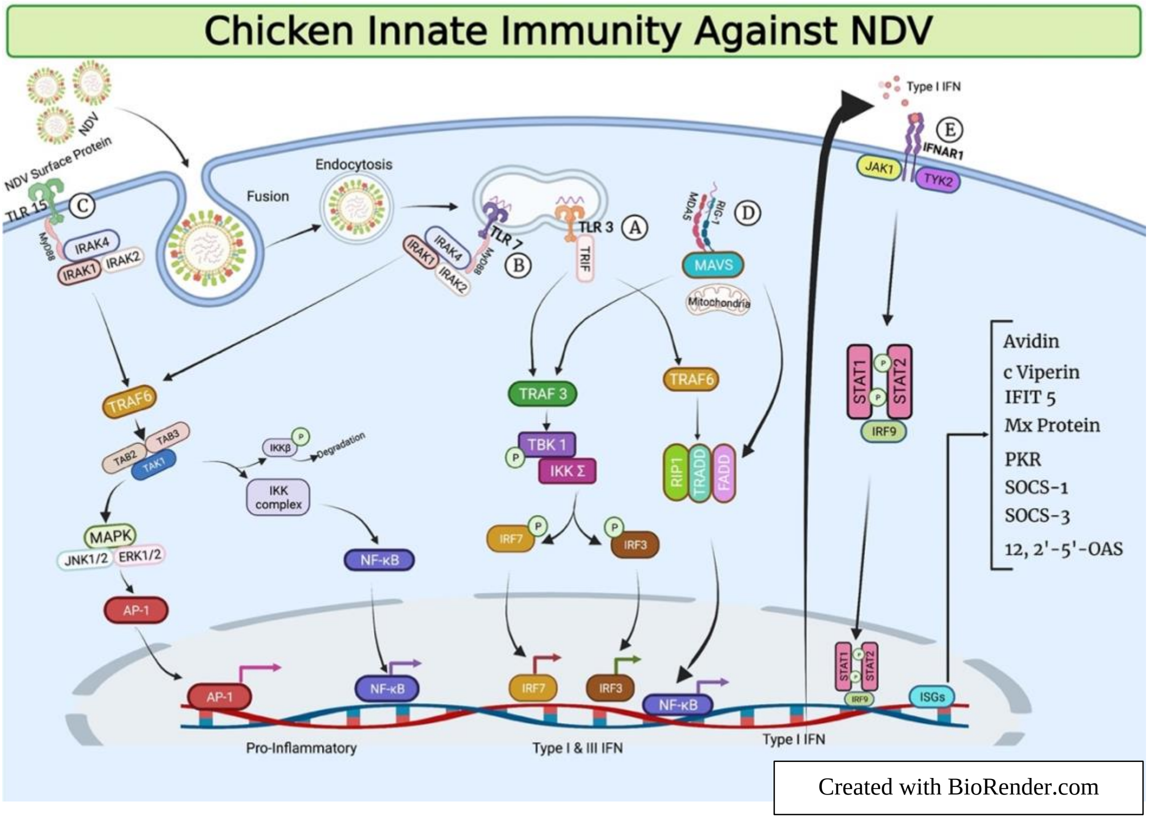

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Figure 1. 1. Chicken innate immunity against NDV at the molecular level. A. TLR3-TRIF-dependent signaling pathway B. TLR7-MyD88-dependent signaling pathway. C. TLR15-MyD88-dependent signaling pathway. D. RLR-MAVS-dependent signaling pathway. E. ISGs signaling pathway.

1. 2. 5. ND Vaccine-Mediated Immunity in Chicken

Briefly, the activation of adaptive immunity begins with innate immunity, which involves dendritic cells (DCs) to capture vaccine antigens and then bring them to drain to lymph nodes, handing over to T cells for B cell activation (Figure 1. 2) [136]. Adjuvant vaccines also have the same capabilities as vaccine antigens in the promotion of adaptive immunity [137]. Adaptive immunity includes cell-mediated immunity (CMI) that utilizes T cells and humoral immunity through B cells activation [138,139]. At vaccination, activating the T cell response requires antigen-presenting cells (APCs), especially DCs and B cells [140,141]. Vaccine antigen/adjuvant must have the ability to provide a “danger signal” so that it can stimulate inflammatory reactions mediated by innate immune response cells (Figure 1. 2A and Figure 1. 2B) [137,142].

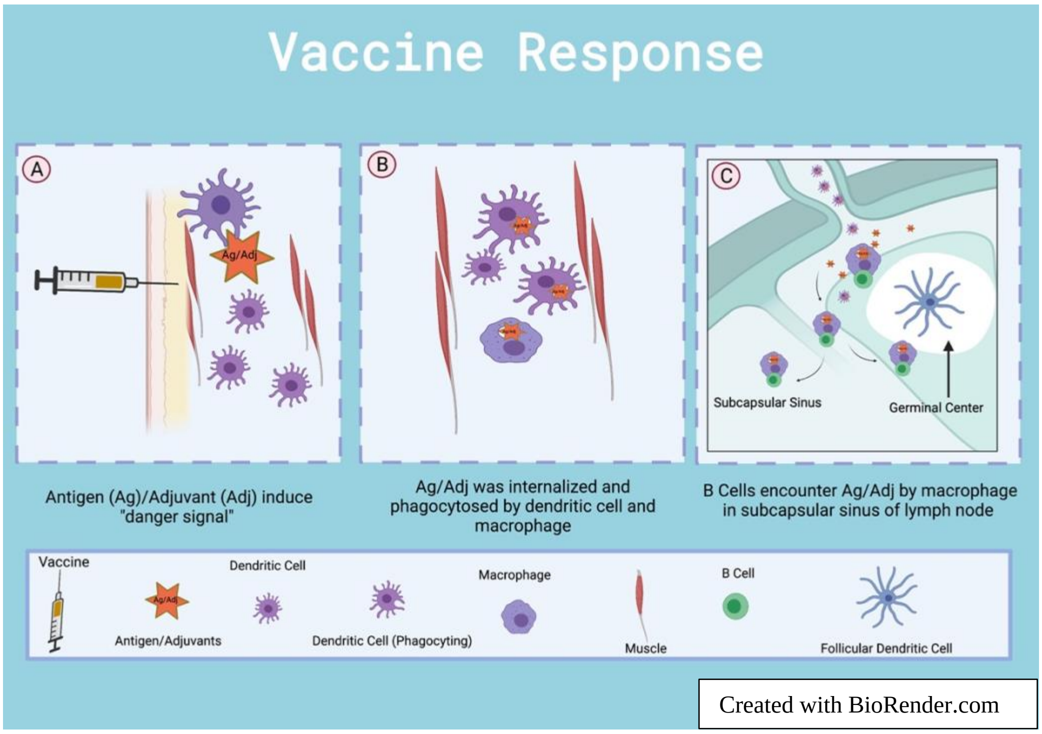

Figure 1. 2. Vaccine response. A. Vaccine antigen/adjuvant vaccine as PAMPs send "danger signal" to attract APCs such as DCs and macrophages. B. Antigen/adjuvant was internalized and phagocytosed by DCs and macrophages. C. B cells encounter antigen/adjuvant by macrophage in subcapsular sinus of lymph node for B cell activation.

T cells are the main part of cell-mediated immunity. It starts with patrolling DCs to capture vaccine antigen at the injection site and then take it to the draining lymph node (LN) for presentation to T cells, activating T cells (Figure 1. 3) [12,143]. This antigen is sensed by DCs using PRR such as TLR7 and TLR 8 recognized single-strand RNA, then carried to draining LN (Figure 1. 3A) [12,144,145]. During migration to LN, the antigen is internalized and phagocyted into small fragments, then displayed on the cell surface for presentation to T helper cells via major histocompatibility complex (MHC) [145,143]. DC presents the fragmented peptide of vaccine antigen either to CD4+ T cell via MHC class II and CD8+ T cells via MHC class I, depending on the size of peptide amino acids (Figure 1. 3B) [139]. The presence of IL-2 can cause the proliferation of CD8+ T cells, producing IFN-γ and TNF-α, and killing the infected cells (Figure 1. 3F/3G/3H) [12]. Meanwhile, the differentiation process of CD4+ T cells into Th1 and Th2 is induced by IFN-γ & IL-12 and IL-4 IL-10, respectively (Figure 1. 3D) [139,146]. Moreover, Th1 also produces IFN-γ, IL-2, TNF, which induces pro-inflammatory cell-mediated immunity [139]. While Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL13 have a significant role in stimulating B cells in producing IgG, IgA, and IgE (Figure 1. 3E) [139]. Naïve B cells encounter antigens in LN marginal zone that are carried by macrophages [138]. B cells can also become APC for CD4+ T helpers by breaking down the antigen into a small peptide and presenting it to T cells via MHC molecules [140].

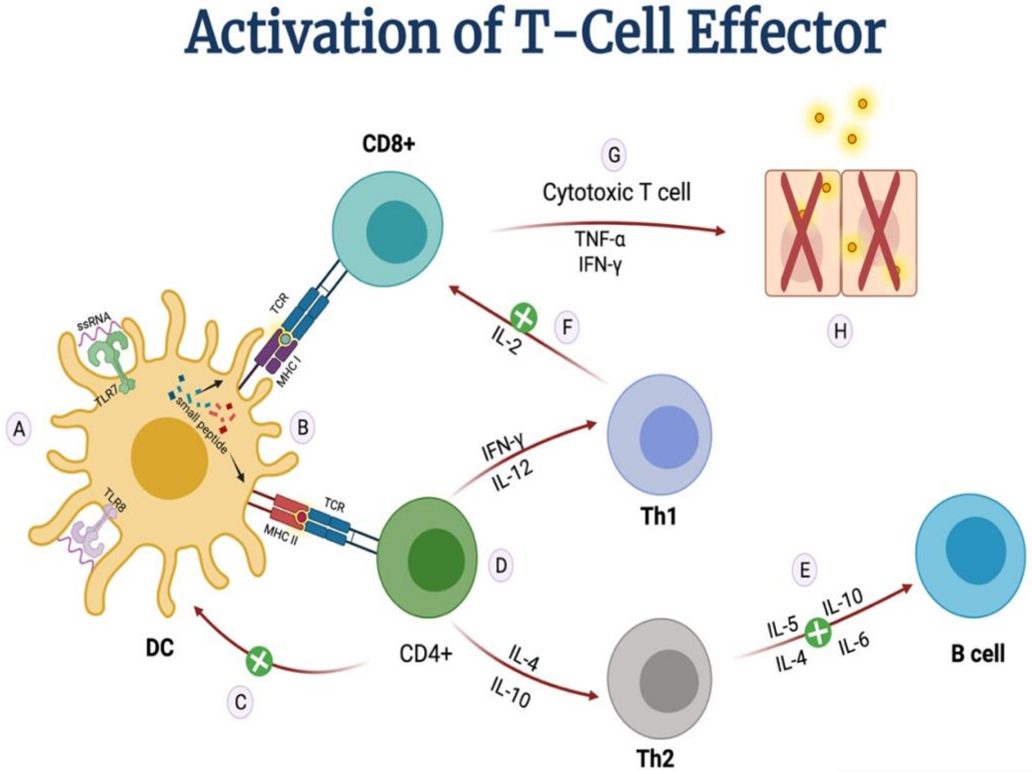

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Figure 1. 3. Activation of the T-cell effector. A. Antigens (ssRNA) are captured by TLR7 and TLR8 on the DC surface and phagocyted into small peptides. B. Peptides are presented by MHC class I to CD4+ T cell or MHC class II to CD8+ T cell. C. Activated CD4+ T cell provides activation signals to DC. D. Differentiation process of CD4+ T cells either into Th1 mediated by IFN-γ and IL-12 or Th2 mediated by IL-4 and IL-10. E. Th2 release cytokine such as IL-6 to proliferate B cells F. Th1 release IL-2 for inducing CD8+ T cell proliferation. H. Infected cells are killed by cytotoxic cells, which are differentiated from CD8+ T cells.

Activation of B cells by involving a T cell-dependent response induces the proliferation of B cells into antibody-secreting plasma cells or memory B cells [147]. Differentiation of B cells into plasma and memory B cells occurs in extrafollicular and germinal center reactions, respectively [12]. In the extrafollicular reaction, naive B cells from the bone marrow capture antigens using IgM receptor binding (Figure 1. 4A). Immature B cells express intact IgM, which plays a role in the autoreactivity test for eliminating B cells that are reactive to “self” protein in the body [138,148]. Activated B cell produces CCR7, a chemokine that brings ag-specific B cells out of the T-cell zone lymph node or extrafollicular and exposed with T helper (Figure 1. 4B/4C) [149]. As a result, B cells are differentiated into short-lived plasma cells that produce low-affinity antibodies (Figure 1. 4D) [150]. In contrast with the extrafollicular reaction, the germinal center (GC) reaction involves T follicular helpers (Tfh) cells activation and proliferation of antigen-specific B cells into long-lived plasma cells and memory B cells [147,151]. B cells will develop into B cell blasts and become part of the follicular DC (FDC), creating a germinal center (Figure 1. 4E) [152]. In addition, the GC consists of Tfh cells and macrophages. The proliferation of B cells to form long-term humoral immunity stimulated by FDC, Tfh cells, and IL-21 (Figure 1. 4F) [153-155]. This proliferation is associated with two significant events, namely the shift of IgM toward IgG, IgA, and IgE and the maturation of the affinity of B cells for their specific antigen. Then plasma cells activated in GC go to bone marrow (BM) for resting in terms of proliferation [153,156]. The duration of the antibody response in the body depends on the number of long-lived plasma cells stimulated [157]. Meanwhile, memory B cells do not produce antibodies but can turn quickly into plasma cells and produce high-affinity antibodies when there is re-infection [157,158].

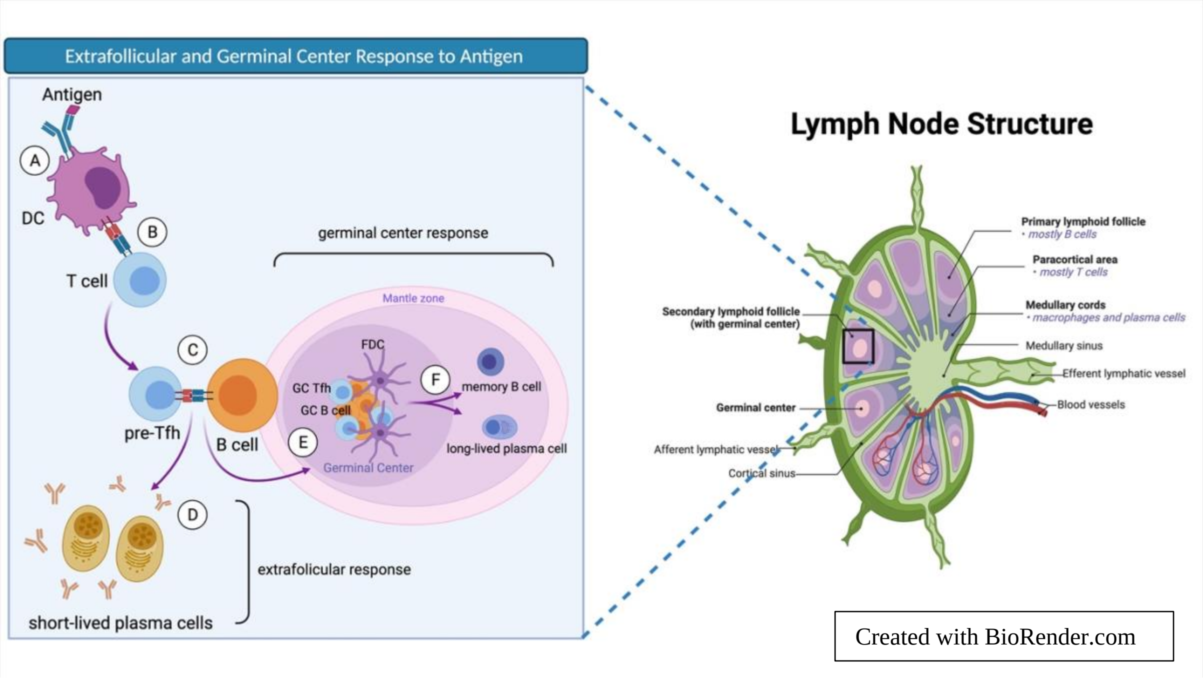

Figure 1. 4. Extrafollicular and germinal center response to antigen vaccine. A. Antigen reaches lymph node and encounters dendritic cell (DC). B. Antigen is phagocyted into a small peptide and presented to the T cell for its activation. C. T helper cell triggers B cell activation in mantle zone or marginal zone. D. In extrafollicular response, some B cells differentiate into a short-lived plasma cell and produce low-affinity antibodies. E. B cells, T follicular helper, and follicular dendritic cell (FDC) initiate the germinal center reaction. F. Germinal center reaction produces memory B cells and long-lived plasma cells, which provide high-affinity antibodies.

Based on the review conducted by Ike et al. (2021), the types of NDV vaccines that are often used worldwide are live and non-live vaccines, which have different abilities in stimulating an immune response in chickens [57]. One of the advantages of the NDV live vaccine is that the price is lower than the inactive vaccine with easy application [159]. One example of a live vaccine still being used today is lentogenic B1 and LaSota strains because they still provide good protection as long as they are used correctly [10,160]. However, how NDV vaccines stimulate the immune response is not as comprehensive as for human vaccines.

NDV Live vaccine has the advantage of inducing cell-mediated and humoral antibodies [66,161]. A few days after being vaccinated with the live vaccine, cell-mediated immunity (CMI) was detected in chickens where CMI reduced viral shedding in the NDV challenge [162]. Another study showed that the live NDV vaccine activates CMI by assessing chicken IFN-γ (chiIFN-γ) [163]. In addition to cell-mediated immunity, especially CD8+ T cells, G7M NDV live vaccine can induce homologous antibodies [66]. IgY and IgA antibodies are generated by VG/GA NDV live vaccine [161]. Live vaccine from LaSota or Roakin strain forms IgM, IgG, and IgA started on the fourth day, while the inactivated vaccine was only developed on the 14th day [164]. Another study showed that the NDV inactive vaccine still can activate CMI, especially CD4+ T cells but takes a longer time [163]. In addition, IFNγ and IL -6 were increased since day 21 after chickens were vaccinated with inactivated NDV vaccine [165].

1. 3. Methods to Achieve the Objectives

1. 3. 1. Transcriptomic Method

The transcriptomic technique was carried out by investigating randomly selected expression of 600 cDNA genes in the human brain in 1991 [110]. The word transcriptomic was first used in the 1990s [166]. In 2006 RNA-sequencing (RNA-seq) began to be applied for transcriptomic profiling of human prostate cancer cDNA using 454 platform [167]. Then the RNA-seq technique has become increasingly popular since the new Illumina technologies platform [168,169].

RNA-seq is one of the techniques for doing transcriptomic. RNA-seq involves high-throughput sequencing where RNA is converted to cDNA to make it more stable at the sequence, and computational methods were to capture and measure transcript in the sample (Figure 1. 5) [170]. In general, preparation before cDNA sequencing is to extract RNA, build cDNA libraries, and sequence using high-throughput platform [171]. RNA extraction consists of disruption of the sample, organic extraction using chloroform, final RNA extraction using ethanol, and measurement of RNA quality and quantity (Figure 1. 6).

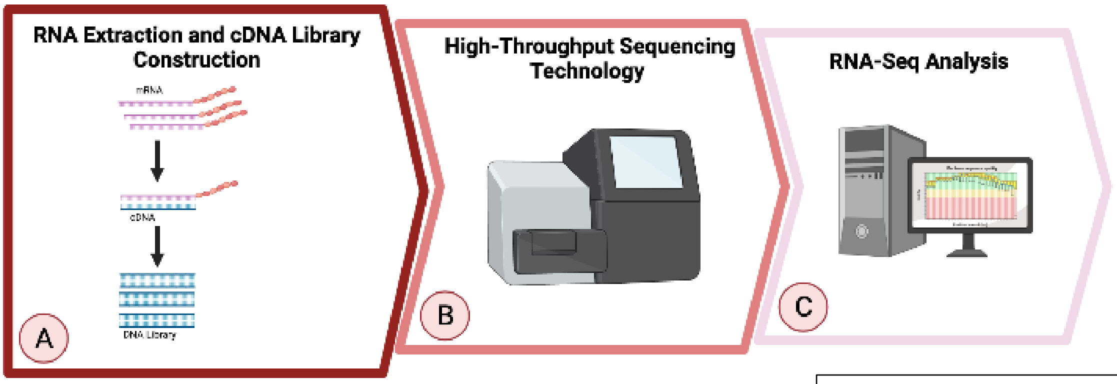

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Figure 1. 5. General scheme of RNA-Seq by using high-throughput sequencing technology. A) RNA Extraction and cDNA library construction. B) High-Throughput Sequencing Technology. C) RNA-Seq Analysis.

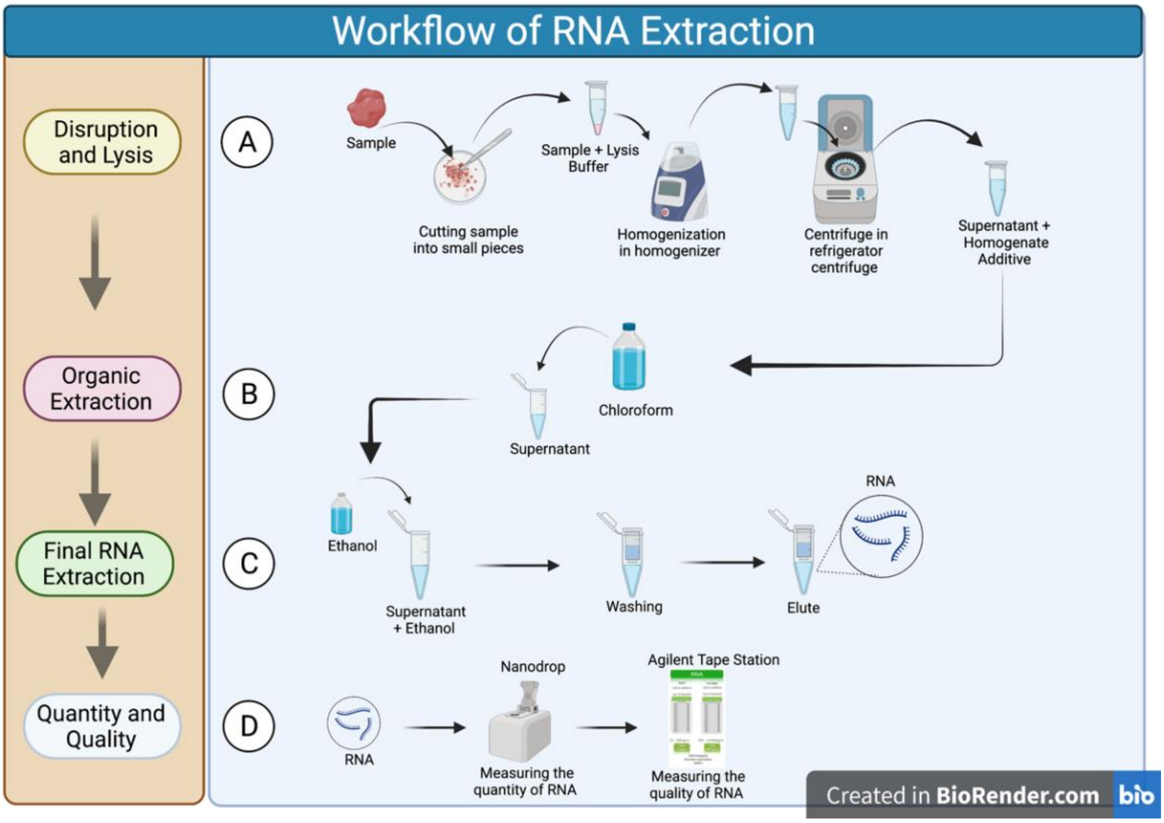

Figure 1. 6. Workflow of RNA extraction. A) Disruption and lysis process. B) Organic extraction with chloroform. C) Final RNA extraction using ethanol. D) RNA quantity and quality using nanodrop and Agilent tape station, respectively.

The process of working on RNA-seq requires some software combined based on the objectives of the experiments. Bioinformaticians have their own developed pipeline. Raw data is the result of high-throughput sequencing. It takes four main steps to analyze the raw data: quality control, alignment process, quantification, and differentially expressed genes (Figure 1. 7) [171,172]. Quality control aims to analyze the quality of the raw data, and the data with bad quality and adapter are removed with a software package such as Trim Galore and AdepterRemoval [173]. The alignment process is where the cleaned sequence read is aligned with the reference genome, were in this study, the chicken reference genome using Hisat2 [174]. For the quantification process, the sequence alignment can be calculated using one of the software packages, namely Ensembl Annotation [175]. Finally, differential expressed genes are measured by normalizing, modeling, and statistically analyzing to identify the up-and down-regulated genes [171].

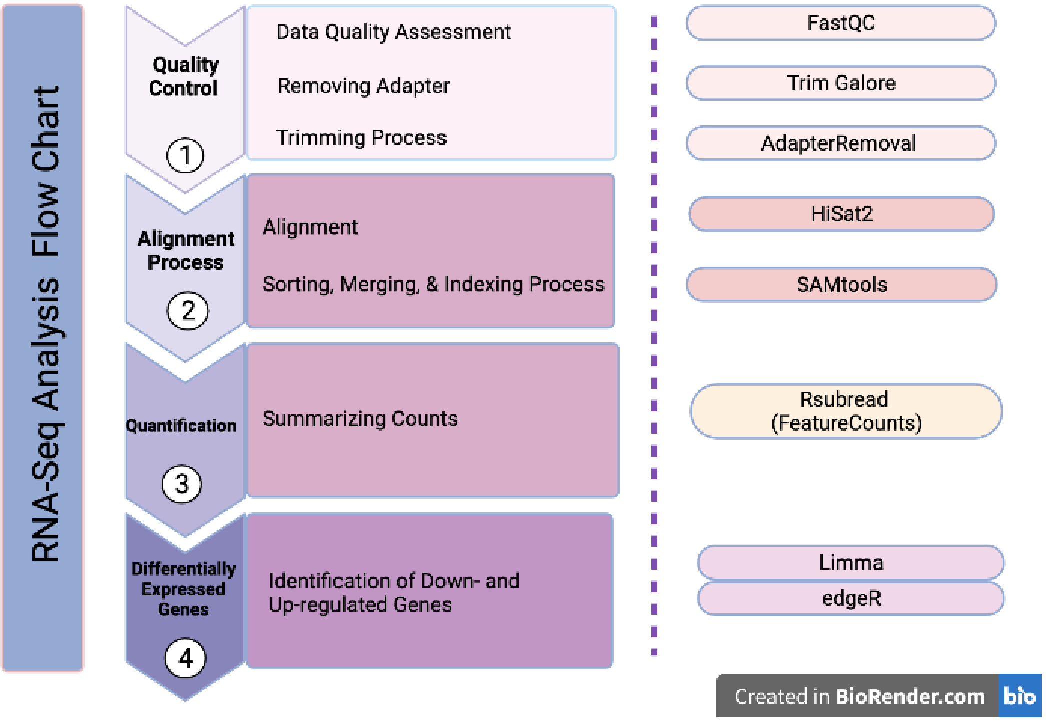

Figure 1. 7. RNA-Seq Analysis Flow Chart. The flow chart contains four stages: quality control, alignment process, quantification, and differentially expressed genes.

Until now, five platforms are often used for RNA-Seq [171,176,177]. The first platform is the 454 made in Roche, Switzerland, where the read length is 700 bp, and the maximum throughput per run is 0.7 Gbp [176]. The second is Illumina, made by Illumina, San Diego, CA, USA (2006) with a read length of 50-300 bp and 900 Gbp of maximum throughput per run [176,177]. In 2008, the third RNA-seq platform was the SOLiD platform made by ThermoFisher Scientific, MA, the USA, where the read length only reached 50 bp where the maximum throughput per run is 320 Gbp [176]. The fourth platform is Ion Torrent from ThermoFisher Scientific, MA, USA (2010), with a read length is 400 bp and maximum throughput per run is 30 Gbp [176,177]. Finally, PacBio is the fifth platform made by Menlo Park, CA, the USA, with a read length of 10,000 bp, and the maximum throughput per run is 2 Gbp [177]. In this study, Illumina NovaSeq 6000 was used for the RNA-seq platform.

1. 3. 2. Haemagglutination (HA) Test and Haemagglutination Inhibition (HI) Test

HA and HI tests are serological tests. HA plays a role in determining viral titers where the hemagglutinin/neuraminidase protein from the virus binds to receptors on red blood cells (RBC) and forms a clumping corresponding to hemagglutination. While the HI test determines the antibody titer in which the antibody attaches to the virus so that the virus does not have a chance to clump the RBC (Figure 1. 8). This method is relatively affordable and can be used on enveloped viruses where the surface of the viral hemagglutinin protein such as NDV.

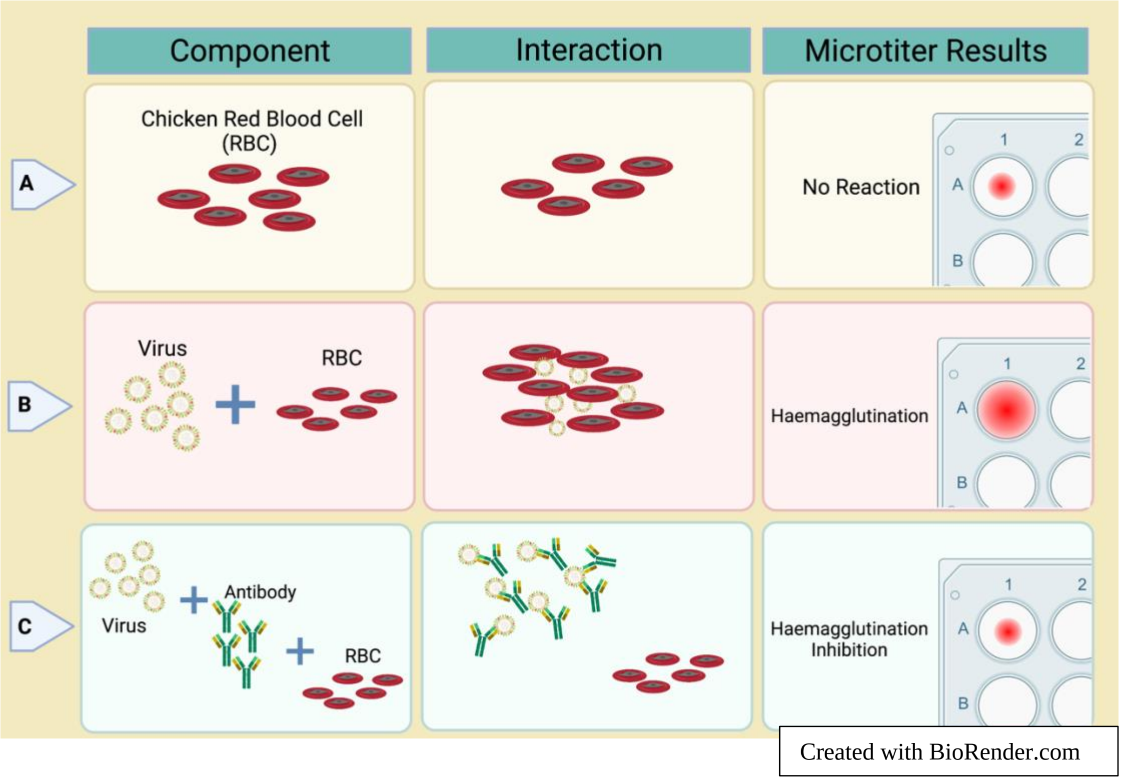

Figure 1. 8. Haemagglutination (HA) Test and Haemagglutination Inhibition (HI) Test. A. No reaction result is a negative control where only RBC settles to the bottom of the well. B. Hemagglutination result shows RBCs are clumped by virus and spread dispersedly at the bottom of the well. C. Hemagglutination Inhibition result shows that RBC settles in one point at the well bottom. This is because the virus has been previously bound by antibodies so that there is no chance for the virus to clump the RBC.

In the HA test, the virus using the haemagglutinin/neuraminidase protein attaches to the receptor on RBC [178]. This binding causes a clumping known as hemagglutination [179]. The HA method begins with a two-fold dilution of virus dispensed into each well of a 96-well microtiter plate. Then, RBC is added to each well. Finally, choose the last well that has clumping and is used as the HA titer of the virus. As a standard condition, HA unit (HAU) corresponds to 10^4 particle viruses per mL which can cause minimum complete agglutination of the RBC [178,179]. Meanwhile, in this study, the virus titer used was 4 HAU as a standard from FAO [179]. The steps in the HA method are depicted in Figure 1. 9.

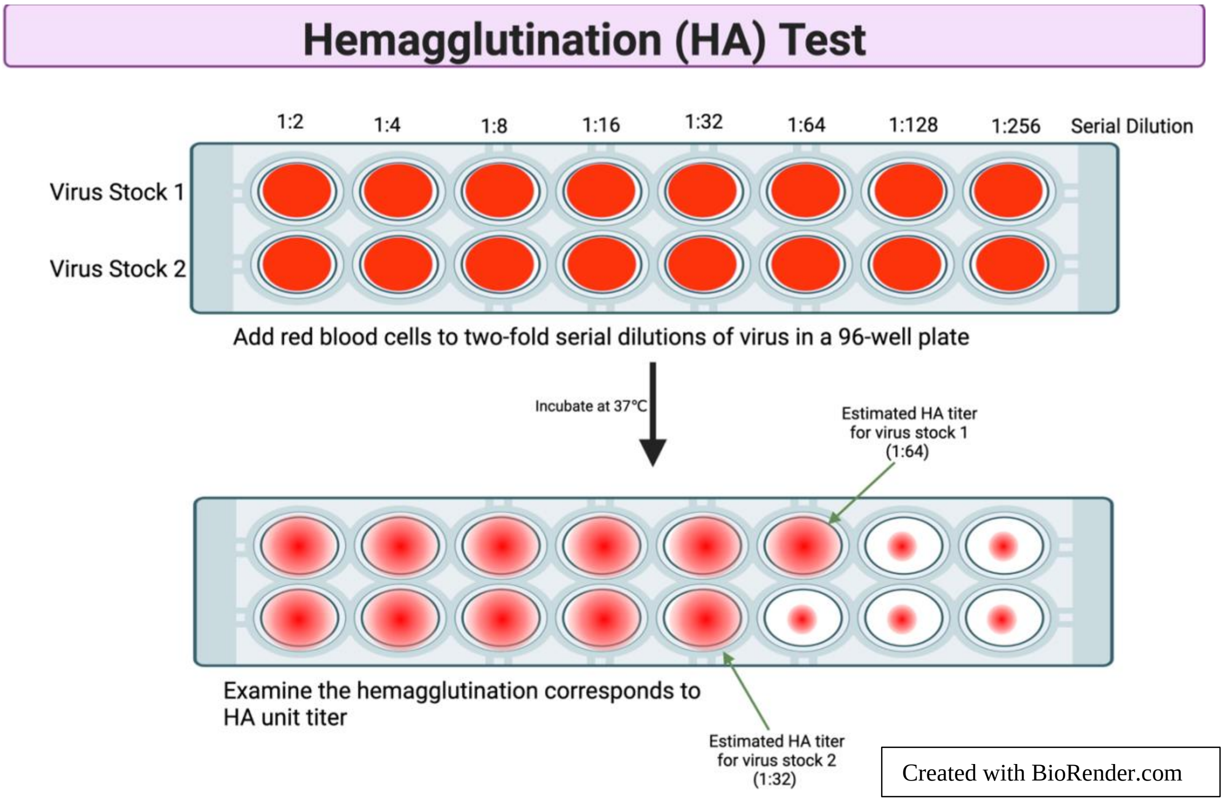

Figure 1. 9. Hemagglutination (HA) test. The second microplate (below) shows that the estimated HA titer for virus stock one is 1:64 while the estimated HA titer for virus stock one is 1:64 while virus stock 2 is 1:32. These results correspond to the HA unit (HAU) titer of the virus.

Hemagglutination Inhibition (HI) test is a serology test used to measure antibody response to viral infection [180]. The HI test utilizes the hemagglutinate (bind) virus to RBC in measuring antibody titer. Specifically, the antibodies contained in the sera will bind to the virus, thereby inhibiting the virus from binding to RBC or known as hemagglutination inhibition. The method depicted in Figure 1. 10 is initiated by a two-fold dilution of serum dispensed into a 96-microplate well. Then, 4 HAU was added to each well. Finally, RBC is added into well.

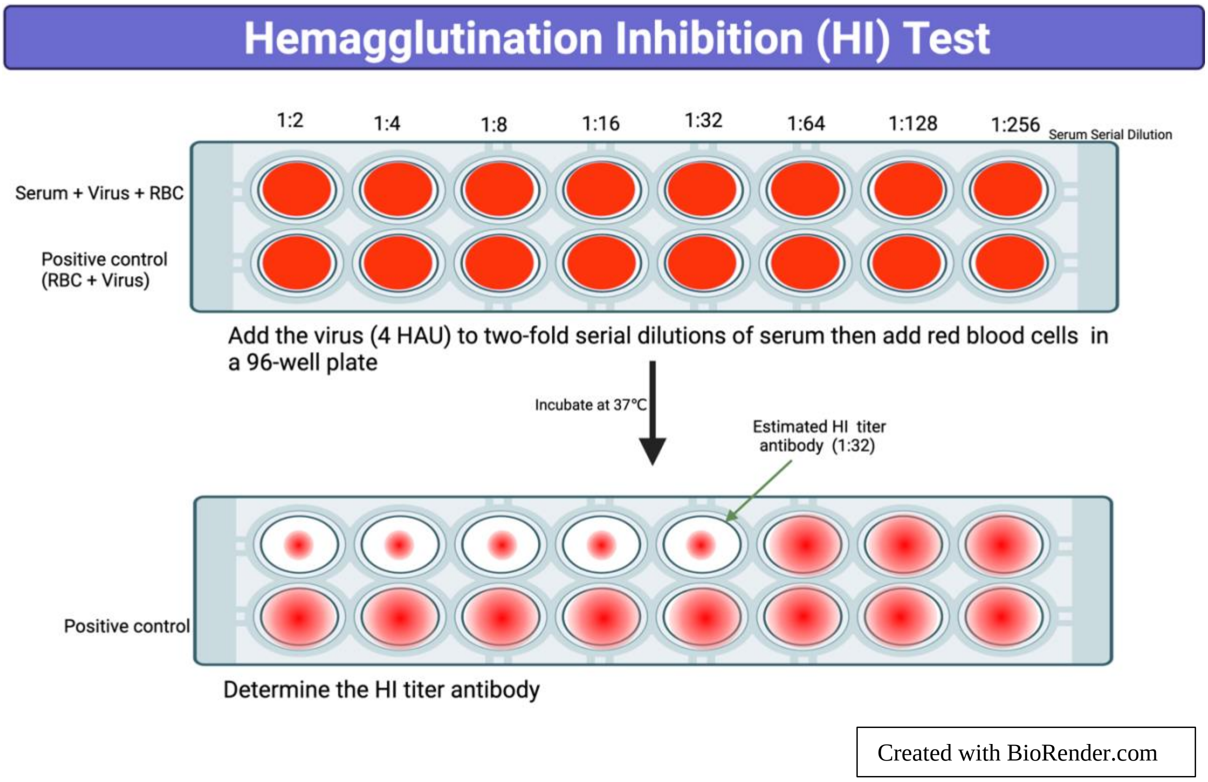

Figure 1. 10. Hemagglutination Inhibition (HI) test. HI titer result in the second microplate shows the estimated HI titer antibody is 1:32. The positive control shows that the virus clumped the RBC.

1. 3. 3. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a method for detecting proteins (antigens or antibodies) using specific antibodies, and this bond is recognized by enzymes and substrate [181]. Moreover, three essential things occur in ELISA: the recognition system that involves immobilization on a solid support, analyzing the substance, and detection system using enzyme and a chromogenic substrate [182-184].

ELISA comprises four types: direct, indirect, sandwich, and competitive ELISA (Figure 1. 11) [182,183,185]. In direct ELISA, the antigen attaches to the solid surface. In this method, only a primary antibody that is also enzyme-conjugated is required to quantify the number of antigens (Figure 1. 11A). Like direct ELISA, indirect ELISA involves a primary antibody that can capture antigen on the solid surface. However, this method involves an enzyme-conjugated secondary antibody to detect an antigen-primary antibody complex (Figure 1. 11B) [182]. While in sandwich ELISA, the solid surface consists of a capture antibody to capture the tested antigen. Then the enzyme-conjugated secondary antibody captures the capture antibody-antigen complex to quantify the number of antigens (Figure 1. 11C) [185]. Finally, competitive ELISA is a method involving antigen-specific antibodies or antibody-specific antigens located on solid surfaces [186]. Then the tested positive sample and enzyme-conjugated antibody or antigen are added together. If the sample contains a positive antigen or antibody, it will attach to the antibody or antigen on the solid surface and compete with enzyme-conjugated antibodies (Figure 1. 11D). Low colorimetric density means positive and vice versa.

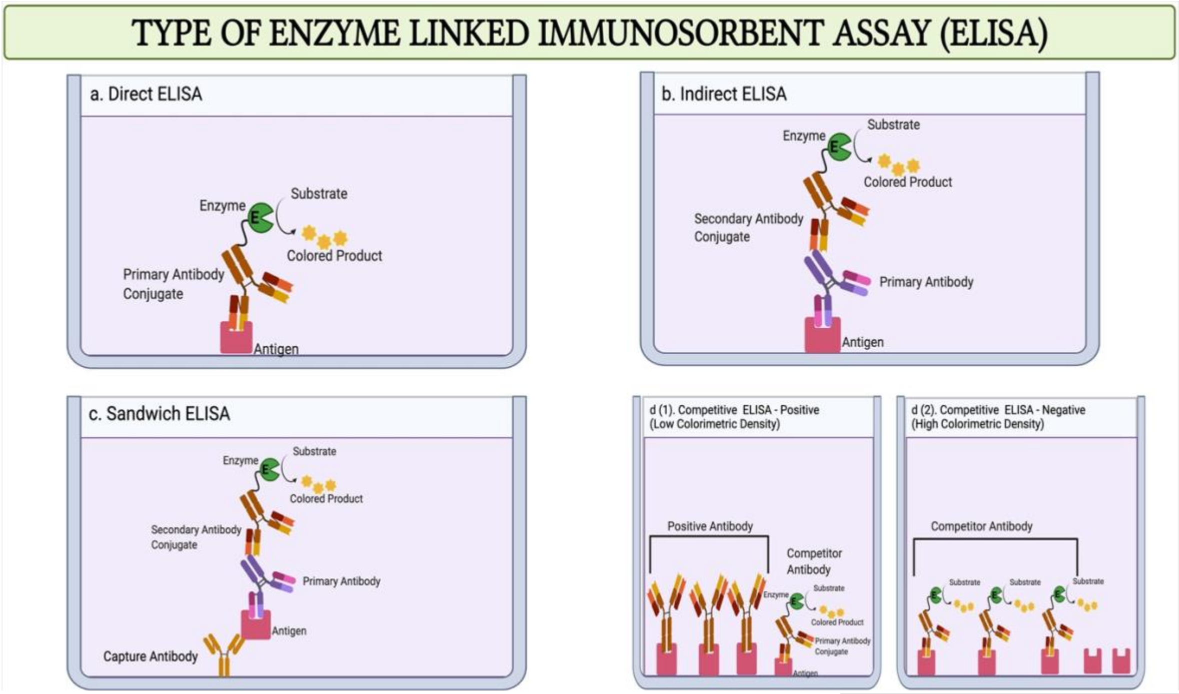

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Figure 1. 11. Types of ELISA. A). Direct ELISA where the antigen is recognized directly by a conjugated detection antibody. B). Indirect ELISA where the antigen is recognized by secondary conjugated detection antibody through the primary antibody. C). Sandwich ELISA where the antigen quantification uses two layers of antibodies (the capture and conjugated detection antibodies). D) Competitive ELISA, positive antibodies compete with enzyme-conjugated antibodies as competitor antibodies to bind with antigen on the solid surface.

1. 3. 4. The Fusion Peptide of Cleavage Site as Antigens

The cleavage site of F protein characteristics in virulent and non-virulent NDV can be used as antigens in ELISA to measure the relevant antibody in vaccinated chicken. The components comprise hydrophobicity and epitopes in the cleavage site of the fusion protein. This study created the fusion peptide from a cleavage site sequence of genotype II and genotype VII.

The F gene has 1792 nucleotides with a single open reading frame encodes 553 amino acids of F protein [187,188]. The fusion protein is synthesized as a precursor fusion protein known as F0 [189]. The activation process of F0 to become F2 and F2, linked by bisulfite binding, involves proteolytic enzyme, protease, or trypsin to cleave the cleavage site of F protein [190]. The sensitivity of F0 protein to protease enzyme depends on the specificity of peptides in the cleavage site, especially in connecting peptides [187,188]. The cleavage site of NDV F protein has two types of residue proteins: single and multi-basic residue [191].

The cleavage site motif of NDV virulent F protein has multi-basic amino acid, ^{112}R/K-R-Q-R/K-R-F^{117}, and protease cleaves arginine residue, especially in the carboxyl site [190-192]. Moreover, arginine at positions 112 and 115 and phenylalanine at positions 117 makes the cleavage process more efficient in virulent NDV [190]. With the low level of hydrophobicity, the cleavage site of F protein NDV virulent is efficiently cleaved by protease enzyme [193]. Protease enzyme is produced all over the host body, while trypsin enzyme is made only in the respiratory and digestive tract [194,195].

In non-virulent NDV F protein, the cleavage site has basic-mono residue with motif ^{112}G-R-Q-G-R-L^{117,} and this sequence is sensitive to trypsin but not to protease [194-196]. With the high level of hydrophobicity, the host protease enzyme has limited access to the cleavage site of non-virulent NDV except at dibasic residue next to the F1 N-terminus [197]. Non-virulent NDV has three regions with high hydrophobicity levels: N terminal signal peptide, N terminus of F1, and a C terminal membrane-spanning region [187].

The antigenic site of the epitope has a function to stimulate the host immune system to produce a specific monoclonal antibody to fight the antigen. There are three terms for antigenic site either in F1 or F2 subunit [189]: site A1-A5 [198]; site I-III [199], which, previously, were I-IV [200]; and site 1 and 2 [201]. Moreover, only in the cleavage site of F protein in virulent NDV has the antigenic site, especially in the K-R-F motif [202].

1. 3. 5. Whole Genome Sequencing

The whole-genome sequence is used to investigate entire DNA sequences, including identifying large structural rearrangements, balanced translocations, and noncoding regions of DNA [203]. In addition, this approach can be applied to viruses such as NDV [204]. Briefly, the steps of performing whole-genome sequences are cDNA preparation, using high-throughput sequencing technology such as the Illumina MiSeq instrument, and data analysis.

Each bioinformatician has a different pipeline in processing the raw data. This study uses contigs assembly, scaffolds extraction, mapping scaffolds to existing NDV sequences, and genome annotation (Figure 1. 12). Phylogenic tree construction is applied to know the distance of this new sequence compared to existing NDV sequences in National Center for Biotechnology Information (NCBI).

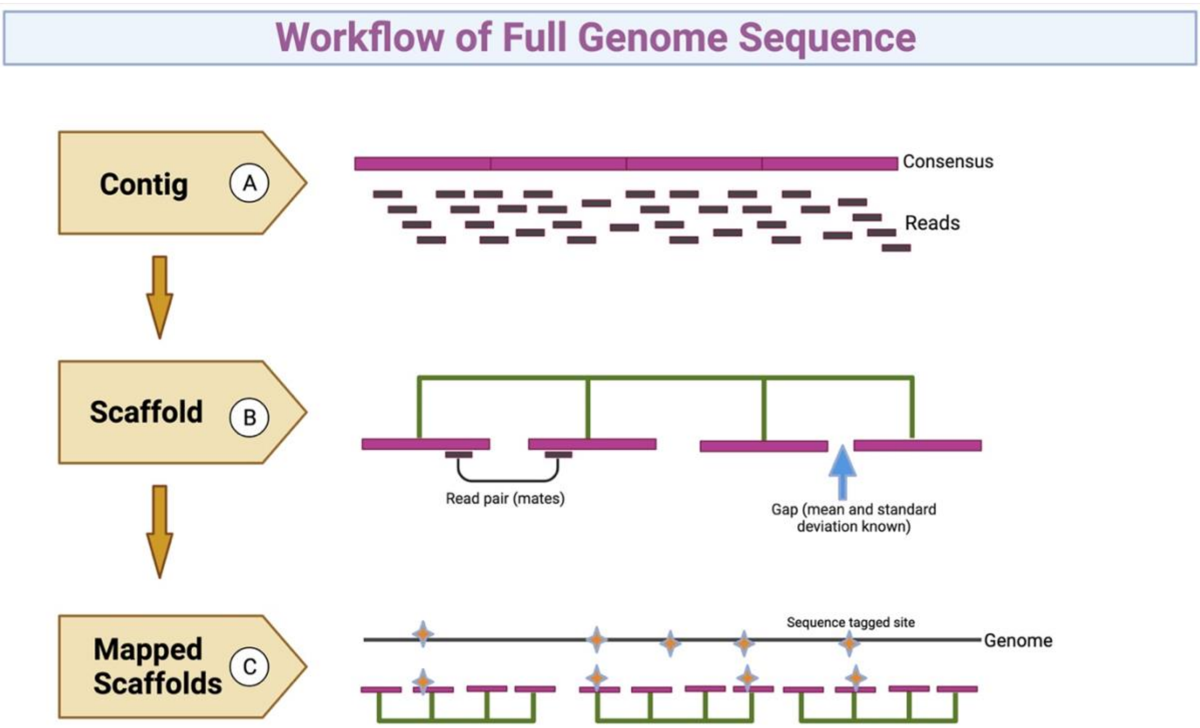

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Figure 1. 12. Mechanism of whole-genome sequence. A. Reads denoted with dark brown line segments are combined to construct contig. B. Contigs are united into scaffolds by pairing end sequences (green line). C. The scaffolds are aligned and mapped to the existing genome using a sequence-tagged site (orange stars).

1. 4. References:

1 Kaleta, E. F. & Baldauf, C. in *Newcastle disease* 197-246 (Springer, 1988).

2 Dimitrov, K. M. *et al.* Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, Genetics and Evolution* **74**, 103917 (2019).

3 Pandarangga, P. *et al.* Full-Genome Sequences of Two Newcastle Disease Virus Strains Isolated in West Java, Indonesia. *Microbiology Resource Announcements* **9** (2020).

4 Lomniczi, B. *et al.* Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). *Archives of virology* **143**, 49-64 (1998).

5 Kim, S.-H. *et al.* (Am Soc Microbiol, 2012).

6 Farhid Hemmatzadeh, M. M. A., Esmaeil Ebrahimie, Simson Tarigan, Mohammad Indro Cahyono. Molecular characterisation of newly emerged Newcastle disease viruses in Indonesia (Australian Centre for International Agricultural Research (ACIAR), Canbera, Australia, 2016).

7 Xiao, S. *et al.* (Am Soc Microbiol, 2012).

8 Hu, S. *et al.* A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. *Vaccine* **27**, 904-910 (2009).

9 Hu, Z. *et al.* Generation of a genotype VII Newcastle disease virus vaccine candidate with high yield in embryonated chicken eggs. *Avian diseases* **55**, 391-397 (2011).

10 Cornax, I., Miller, P. J. & Afonso, C. L. Characterization of live LaSota vaccine strain–induced protection in chickens upon early challenge with a virulent Newcastle disease virus of heterologous genotype. *Avian diseases* **56**, 464-470 (2012).

11 Pulendran, B., Li, S. & Nakaya, H. I. Systems vaccinology. *Immunity* **33**, 516-529 (2010).

12 Siegrist, C.-A. Vaccine immunology. *Vaccines* **5**, 17-36 (2008).

13 Rima, B. *et al.* ICTV Virus Taxonomy Profile: Paramyxoviridae. *Journal of General Virology*, jgv001328 (2019).

14 Czeglédi, A. *et al.* Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. *Virus research* **120**, 36-48 (2006).

15 Lamb, R. A. & Parks, G. D. in *Fields Virology: Sixth Edition* (Wolters Kluwer Health Adis (ESP), 2013).

16 Lamb, R. A. Paramyxoviridae: the viruses and their replication. *Fields virology* (2001).

17 Steward, M., Vipond, I. B., Millar, N. S. & Emmerson, P. T. RNA editing in Newcastle disease virus. *Journal of General Virology* **74**, 2539-2547 (1993).

18 Mast, J. & Demeestere, L. Electron tomography of negatively stained complex viruses: application in their diagnosis. *Diagnostic pathology* **4**, 5 (2009).

19 Li, J. & Zhang, Y. in *Methylation-From DNA, RNA and Histones to Diseases and Treatment* (IntechOpen, 2012).

20 Muñoz-Barroso, I., Cobaleda, C., Zhadan, G., Shnyrov, V. & Villar, E. Dynamic properties of Newcastle Disease Virus envelope and their relations with viral hemagglutinin-neuraminidase membrane glycoprotein. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1327**, 17-31 (1997).

21 ICTV. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. (Academic Press, Waltham, MA, 2012).

22 Miller, P., Koch, G. & Suarez, D. L. in *Diseases of poultry* (eds David E. Swayne & John R. Glisson) 89-138 (Wiley-Blackwell, 2013).

23 Crennell, S., Takimoto, T., Portner, A. & Taylor, G. Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. *Nature Structural & Molecular Biology* **7**, 1068-1074 (2000).

24 Lamb, R. A. & Kolakofsky, D. in *Fundamental virology* (eds D. M. Knipe & P. M. Howley) (Lippincott Williams & Wilkins, Philadelphia; USA, 2001).

25 de Leeuw, O. S., Koch, G., Hartog, L., Ravenshorst, N. & Peeters, B. P. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin–neuraminidase protein. *Journal of General Virology* **86**, 1759-1769 (2005).

26 Morrison, T. G. Structure and function of a paramyxovirus fusion protein. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1614**, 73-84 (2003).

27 Römer-Oberdörfer, A., Werner, O., Veits, J., Mebatsion, T. & Mettenleiter, T. C. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. *Journal of General Virology* **84**, 3121-3129 (2003).

28 Mebatsion, T., Verstegen, S., Vaan, L. T. C. d., Römer-Oberdörfer, A. & Schrier, C. C. A recombinant newcastle disease virus with low-level V protein expression is immunogenic and lacks pathogenicity for chicken embryos. *Journal of virology* **75**, 420-428 (2001).

29 Huang, Z., Krishnamurthy, S., Panda, A. & Samal, S. K. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. *Journal of virology* **77**, 8676-8685 (2003).

30 Miller, P. J. *et al.* Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Infection, genetics and evolution* **29**, 216-229 (2015).

31 Diel, D. G. *et al.* Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* **12**, 1770-1779, doi:10.1016/j.meegid.2012.07.012 (2012).

32 Miller, P. J., Decanini, E. L. & Afonso, C. L. Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* **10**, 26-35, doi:10.1016/j.meegid.2009.09.012 (2010).

33 Khan, T. A. *et al.* Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. *Journal of clinical microbiology* **48**, 1892-1894, doi:10.1128/JCM.00148-10 (2010).

34 Liu, X., Wan, H., Ni, X., Wu, Y. & Liu, W. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. *Archives of virology* **148**, 1387-1403 (2003).

35 Tsai, H.-J. *et al.* Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. *Veterinary microbiology* **104**, 19-30 (2004).

36 Wang, Z. *et al.* Genotyping of Newcastle disease viruses isolated from 2002 to 2004 in China. *Annals of the New York Academy of Sciences* **1081**, 228-239 (2006).

37 Yu, L., Wang, Z., Jiang, Y., Chang, L. & Kwang, J. Characterization of newly emerging Newcastle disease virus isolates from the People's Republic of China and Taiwan. *Journal of clinical microbiology* **39**, 3512-3519, doi:10.1128/JCM.39.10.3512-3519.2001 (2001).

38 Alexander, D. J. Newcastle disease. *British poultry science* **42**, 5-22 (2001).

39 Afonso, C. L., Miller, P., Haddas, R. & Simanov, L. Emergence of new sub-genotypes of virulent Newcastle disease virus with panzootic features. *Infection, Genetics and Evolution* **In Press** (2015).

40 Alexander, D. J. Gordon Memorial Lecture. Newcastle disease. *British poultry science* **42**, 5-22, doi:10.1080/713655022 (2001).

41 Hanson, R. P. World wide spread of viscerotropic Newcastle disease. *Proceedings of the United States Health Association* **76**, 276-279 (1972).

42 Lomniczi, B. *et al.* Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). *Archives Of Virology* **143**, 49-64 (1998).

43 Kaleta, E. F., Alexander, D. J. & Russell, P. H. The first isolation of the avian pmv-1 virus responsible for the current panzootic in pigeons ? *Avian Pathology* **14**, 553 (1985).

44 Perozo, F., Marcano, R. & Afonso, C. L. Biological and phylogenetic characterization of a genotype VII Newcastle disease virus from Venezuela: efficacy of field vaccination. *Journal of clinical microbiology* **50**, 1204-1208, doi:10.1128/JCM.06506-11 (2012).

45 PLAN, A. V. E. Disease Strategy Newcastle disease. (2010).

46 Gould, A. R. *et al.* Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998–2000. *Virus research* **77**, 51-60 (2001).

47 Guèye, E. Employment and income generation through family poultry in low-income food-deficit countries. *World's Poultry Science Journal* **58**, 541-557 (2002).

48 Xiao, S. *et al.* Complete genome sequences of Newcastle disease virus strains circulating in chicken populations of Indonesia. *Journal of virology* **86**, 5969-5970 (2012).

49 Kapczynski, D. R. & King, D. J. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* **23**, 3424-3433 (2005).

50 Goldhaft, T. M. Guest editorial: Historical note on the origin of the LaSota strain of Newcastle disease virus. *Avian diseases* **24**, 297-301 (1980).

51 Kim, L. M., King, D. J., Suarez, D. L., Wong, C. W. & Afonso, C. L. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. *Journal of clinical microbiology* **45**, 1310-1314 (2007).

52 Ababneh, M. M., Dalab, A. E., Alsaad, S. R., Al-Zghoul, M. B. & Al-Natour, M. Q. Molecular characterization of a recent Newcastle disease virus outbreak in Jordan. *Research in veterinary science* **93**, 1512-1514 (2012).

53 Radwan, M. M., Darwish, S. F., El-Sabagh, I. M., El-Sanousi, A. A. & Shalaby, M. A. Isolation and molecular characterization of Newcastle disease virus genotypes II and VIId in Egypt between 2011 and 2012. *Virus Genes* **47**, 311-316.

54 Yi, J., Liu, C., Chen, B. & Wu, S. Molecular characterization of a virulent genotype VIId strain of Newcastle disease virus from farmed chickens in Shanghai. *Avian Dis* **55**, 279-284 (2011).

55 Abolnik, C. *et al.* A phylogenetic study of South African Newcastle disease virus strains isolated between 1990 and 2002 suggests epidemiological origins in the Far East. *Archives of Virology* **149**, 603-619 (2004).

56 Dimitrov, K. M., Afonso, C. L., Yu, Q. & Miller, P. J. Newcastle disease vaccines-A solved problem or a continuous challenge? *Vet Microbiol* **206**, 126-136, doi:10.1016/j.vetmic.2016.12.019 (2017).

57 Ike, A. C. *et al.* Towards Improved Use of Vaccination in the Control of Infectious Bronchitis and Newcastle Disease in Poultry: Understanding the Immunological Mechanisms. *Vaccines* **9**, 20 (2021).

58 Huang, Z., Elankumaran, S., Yunus, A. S. & Samal, S. K. A recombinant Newcastle disease virus (NDV) expressing VP2 protein of infectious bursal disease virus (IBDV) protects against NDV and IBDV. *Journal of virology* **78**, 10054-10063 (2004).

59 Yu, Q. *et al.* Protection by recombinant Newcastle disease viruses (NDV) expressing the glycoprotein (G) of avian metapneumovirus (aMPV) subtype A or B against challenge with virulent NDV and aMPV. *World Journal of Vaccines* **2013** (2013).

60 Miller, P. J., King, D. J., Afonso, C. L. & Suarez, D. L. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. *Vaccine* **25**, 7238-7246 (2007).

61 Perozo, F., Villegas, P., Dolz, R., Afonso, C. L. & Purvis, L. B. The VG/GA strain of Newcastle disease virus: mucosal immunity, protection against lethal challenge and molecular analysis. *Avian Pathology* **37**, 237-245 (2008).

62 Xiao, S. *et al.* Generation by reverse genetics of an effective, stable, live-attenuated Newcastle disease virus vaccine based on a currently circulating, highly virulent Indonesian strain. *PloS one* **7**, e52751 (2012).

63 Liu, M.-M. *et al.* Generation by reverse genetics of an effective attenuated Newcastle disease virus vaccine based on a prevalent highly virulent Chinese strain. *Biotechnology letters* **37**, 1287-1296 (2015).

64 Sun, M. *et al.* Generation and evaluation of a genetically attenuated Newcastle disease virus rGM-VIIm as a genotype-matched vaccine. *Virus genes* **53**, 35-43 (2017).

65 Yadav, K. *et al.* Generation and evaluation of a recombinant Newcastle disease virus strain R2B with an altered fusion protein cleavage site as a vaccine candidate. *Microbial pathogenesis* **118**, 230-237 (2018).

66 Ji, Y. *et al.* A novel genotype VII Newcastle disease virus vaccine candidate generated by mutation in the L and F genes confers improved protection in chickens. *Veterinary microbiology* **216**, 99-106 (2018).

67 Izquierdo-Lara, R., Chumbe, A., Calderón, K., Fernández-Díaz, M. & Vakharia, V. N. Genotype-matched Newcastle disease virus vaccine confers improved protection against genotype XII challenge: The importance of cytoplasmic tails in viral replication and vaccine design. *PloS one* **14**, e0209539 (2019).

68 Bello, M. B. *et al.* Development of an effective and stable genotype-matched live attenuated Newcastle disease virus vaccine based on a novel naturally recombinant Malaysian isolate using reverse genetics. *Vaccines* **8**, 270 (2020).

69 Li, S.-y. *et al.* A class I lentogenic newcastle disease virus strain confers effective protection against the prevalent strains. *Biologicals* **63**, 74-80 (2020).

70 Liu, J. *et al.* Effects of the HN antigenic difference between the vaccine strain and the challenge strain of Newcastle disease virus on virus shedding and transmission. *Viruses* **9**, 225 (2017).

71 Basavarajappa, M. K. *et al.* A recombinant Newcastle disease virus (NDV) expressing infectious laryngotracheitis virus (ILTV) surface glycoprotein D protects against highly virulent ILTV and NDV challenges in chickens. *Vaccine* **32**, 3555-3563 (2014).

72 Kapczynski, D. R., Afonso, C. L. & Miller, P. J. Immune responses of poultry to Newcastle disease virus. *Dev Comp Immunol* **41**, 447-453, doi:10.1016/j.dci.2013.04.012 (2013).

73 Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology* **11**, 373 (2010).

74 Yoneyama, M. & Fujita, T. RIG-I family RNA helicases: cytoplasmic sensor for antiviral innate immunity. *Cytokine & growth factor reviews* **18**, 545-551 (2007).

75 Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805-820 (2010).

76 Jensen, S. & Thomsen, A. R. Sensing of RNA viruses: a review of innate immune receptors involved in recognizing RNA virus invasion. *Journal of virology* **86**, 2900-2910 (2012).

77 Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**, 637-650 (2011).

78 Juul-Madsen, H. R., Viertlboeck, B., Smith, A. L. & Göbel, T. W. Avian innate immune responses. *Avian immunology*, 129-158 (2008).

79 Boyd, Y., Goodchild, M., Morroll, S. & Bumstead, N. Mapping of the chicken and mouse genes for toll-like receptor 2 (TLR2) to an evolutionarily conserved chromosomal segment. *Immunogenetics* **52**, 294-298 (2001).

80 Fukui, A. *et al.* Molecular cloning and functional characterization of chicken toll-like receptors A single chicken toll covers multiple molecular patterns. *Journal of Biological Chemistry* **276**, 47143-47149 (2001).

81 Leveque, G. *et al.* Allelic variation in TLR4 is linked to susceptibility to Salmonella enterica serovar Typhimurium infection in chickens. *Infection and immunity* **71**, 1116-1124 (2003).

82 Iqbal, M. *et al.* Identification and functional characterization of chicken toll-like receptor 5 reveals a fundamental role in the biology of infection with Salmonella enterica serovar typhimurium. *Infection and immunity* **73**, 2344-2350 (2005).

83 Philbin, V. J. *et al.* Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology* **114**, 507-521 (2005).

84 Roach, J. C. *et al.* The evolution of vertebrate Toll-like receptors. *Proceedings of the National Academy of Sciences* **102**, 9577-9582 (2005).

85 Cheng, J. *et al.* Toll-like receptor 3 inhibits Newcastle disease virus replication through activation of pro-inflammatory cytokines and the type-1 interferon pathway. *Archives of virology* **159**, 2937-2948 (2014).

86 Paulmann, D. *et al.* NF-κB activation induced by hepatitis A virus and Newcastle disease virus occurs by different pathways depending on the structural pattern of viral nucleic acids. *Archives of virology* **159**, 1723-1733 (2014).

87 Rue, C. A. *et al.* Virulent Newcastle disease virus elicits a strong innate immune response in chickens. *J Gen Virol* **92**, 931-939, doi:10.1099/vir.0.025486-0 (2011).

88 Kang, Y. *et al.* Host innate immune responses of ducks infected with Newcastle disease viruses of different pathogenicities. *Frontiers in microbiology* **6**, 1283 (2015).

89 Wilden, H., Fournier, P., Zawatzky, R. & Schirrmacher, V. Expression of RIG-I, IRF3, IFN-β and IRF7 determines resistance or susceptibility of cells to infection by Newcastle Disease Virus. *International journal of oncology* **34**, 971-982 (2009).

90 Liu, W. Q. *et al.* The different expression of immune-related cytokine genes in response to velogenic and lentogenic Newcastle disease viruses infection in chicken peripheral blood. *Mol Biol Rep* **39**, 3611-3618, doi:10.1007/s11033-011-1135-1 (2012).

91 Chen, H. & Jiang, Z. The essential adaptors of innate immune signaling. *Protein & cell* **4**, 27-39 (2013).

92 García-Sastre, A. & Biron, C. A. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* **312**, 879-882 (2006).

93 Matsumoto, M. & Seya, T. TLR3: interferon induction by double-stranded RNA including poly (I: C). *Advanced drug delivery reviews* **60**, 805-812 (2008).

94 Wang, Y. *et al.* Molecular characterization, expression of chicken TBK1 gene and its effect on IRF3 signaling pathway. *PLoS One* **12**, e0177608 (2017).

95 Oganesyan, G. *et al.* Critical role of TRAF3 in the Toll-like receptor-dependent and-independent antiviral response. *Nature* **439**, 208-211 (2006).

96 Lin, D. *et al.* Induction of USP25 by viral infection promotes innate antiviral responses by mediating the stabilization of TRAF3 and TRAF6. *Proceedings of the National Academy of Sciences* **112**, 11324-11329 (2015).

97 Zhang, T. *et al.* Comparative analysis of early immune responses induced by two strains of Newcastle disease virus in chickens. *MicrobiologyOpen* **8**, e00701 (2019).

98 Li, Y. *et al.* Host avian beta-defensin and toll-like receptor responses of pigeons following infection with pigeon paramyxovirus type 1. *Applied and environmental microbiology* **81**, 6415-6424 (2015).

99 Muzio, M., Ni, J., Feng, P. & Dixit, V. M. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612-1615 (1997).

100 Kawagoe, T. *et al.* Sequential control of Toll-like receptor–dependent responses by IRAK1 and IRAK2. *Nature immunology* **9**, 684 (2008).

101 Qian, Y., Commane, M., Ninomiya-Tsuji, J., Matsumoto, K. & Li, X. IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFκB. *Journal of Biological Chemistry* **276**, 41661-41667 (2001).

102 Horng, T., Barton, G. M. & Medzhitov, R. TIRAP: an adapter molecule in the Toll signaling pathway. *Nature immunology* **2**, 835-841 (2001).

103 Deng, L. *et al.* Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**, 351-361 (2000).

104 Wang, C. *et al.* TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**, 346-351 (2001).

105 Akira, S. & Takeda, K. Toll-like receptor signalling. *Nature reviews immunology* **4**, 499-511 (2004).

106 Jiang, X. *et al.* Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response. *Immunity* **36**, 959-973 (2012).

107 Hou, F. *et al.* MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell* **146**, 448-461 (2011).

108 Michallet, M.-C. *et al.* TRADD protein is an essential component of the RIG-like helicase antiviral pathway. *Immunity* **28**, 651-661 (2008).

109 Wang, Z. *et al.* Interferon regulatory factor 7-(IRF7-) mediated immune response affects Newcastle disease virus replication in chicken embryo fibroblasts. *Acta Veterinaria Hungarica* **62**, 500-511 (2014).

110 Adams, M. D. *et al.* Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* **252**, 1651-1656 (1991).

111 Susta, L. *et al.* Expression of interferon gamma by a highly virulent strain of Newcastle disease virus decreases its pathogenicity in chickens. *Microbial pathogenesis* **61**, 73-83 (2013).

112 Kaiser, P. & Stäheli, P. in *Avian Immunology (Second Edition)* 189-204 (Elsevier, 2014).

113 Sekellick, M. J., FERRANDINO, A. F., HOPKINS, D. A. & MARCUS, P. I. Chicken interferon gene: cloning, expression, and analysis. *Journal of interferon research* **14**, 71-79 (1994).

114 Karpala, A. J. *et al.* Molecular cloning, expression, and characterization of chicken IFN-λ. *journal of interferon & cytokine research* **28**, 341-350 (2008).

115 Turner, M. D., Nedjai, B., Hurst, T. & Pennington, D. J. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **1843**, 2563-2582 (2014).

116 Negishi, H. *et al.* Evidence for licensing of IFN-γ-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. *Proceedings of the National Academy of Sciences* **103**, 15136-15141 (2006).

117 Schmitz, F. *et al.* Interferon-regulatory-factor 1 controls Toll-like receptor 9-mediated IFN-β production in myeloid dendritic cells. *European journal of immunology* **37**, 315-327 (2007).

118 Kishimoto, T. IL-6: from its discovery to clinical applications. *International immunology* **22**, 347-352 (2010).

119 Wang, Y., van Boxel-Dezaire, A. H., Cheon, H., Yang, J. & Stark, G. R. STAT3 activation in response to IL-6 is prolonged by the binding of IL-6 receptor to EGF receptor. *Proceedings of the National Academy of Sciences* **110**, 16975-16980 (2013).

120 Luo, J. *et al.* Recombinant rabies virus expressing interleukin-6 enhances the immune response in mouse brain. *Archives of virology* **163**, 1889-1895 (2018).

121 Santhakumar, D., Rubbenstroth, D., Martinez-Sobrido, L. & Munir, M. Avian interferons and their antiviral effectors. *Frontiers in immunology* **8**, 49 (2017).

122 Pestka, S., Krause, C. D. & Walter, M. R. Interferons, interferon-like cytokines, and their receptors. *Immunological reviews* **202**, 8-32 (2004).

123 Gao, M. *et al.* Evolutional conservation of molecular structure and antiviral function of a type I interferon, IFN-kappa, in poultry. *Developmental & Comparative Immunology* **89**, 44-53 (2018).

124 Ivashkiv, L. B. & Donlin, L. T. Regulation of type I interferon responses. *Nature Reviews Immunology* **14**, 36-49 (2014).

125 Schmeisser, H., Bekisz, J. & Zoon, K. C. New function of type I IFN: induction of autophagy. *Journal of interferon & cytokine research* **34**, 71-78 (2014).

126 Guo, S.-Y., Li, C., Dai, X.-M., Zhao, C. & Chang, W.-S. Distribution of the type I interferon in different organs of chicken digestive system. *Central-European journal of immunology* **39**, 455 (2014).

127 Qu, H. *et al.* The differential antiviral activities of chicken interferon α (ChIFN-α) and ChIFN-β are related to distinct interferon-stimulated gene expression. *PLoS One* **8**, e59307 (2013).

128 Payne, S. Chapter 6—immunity and resistance to viruses. *Viruses; Payne, S., Ed.; Academic Press: Cambridge, MA, USA*, 61-71 (2017).

129 Han, C.-L., Zhang, W., Dong, H.-T., Han, X. & Wang, M. A novel gene of β chain of the IFN-γ receptor of Huiyang chicken: cloning, distribution, and CD assay. *Journal of interferon & cytokine research* **26**, 441-448 (2006).

130 Han, X., Chen, T. & Wang, M. Molecular cloning and characterization of chicken interferon-γ receptor α-chain. *Journal of Interferon & Cytokine Research* **28**, 445-454 (2007).

131 Stanifer, M. L., Pervolaraki, K. & Boulant, S. Differential regulation of type I and type III interferon signaling. *International journal of molecular sciences* **20**, 1445 (2019).

132 Dellgren, C., Gad, H., Hamming, O., Melchjorsen, J. & Hartmann, R. Human interferon-λ3 is a potent member of the type III interferon family. *Genes & Immunity* **10**, 125-131 (2009).

133 Reuter, A. *et al.* Antiviral activity of lambda interferon in chickens. *Journal of virology* **88**, 2835-2843 (2014).

134 Shah, M., Bharadwaj, M., Gupta, A., Kumar, R. & Kumar, S. Chicken viperin inhibits Newcastle disease virus infection in vitro: a possible interaction with the viral matrix protein. *Cytokine* **120**, 28-40 (2019).

135 Zhang, Y. *et al.* Partial antiviral activities detection of chicken Mx jointing with neuraminidase gene (NA) against Newcastle disease virus. *Plos one* **8**, e71688 (2013).

136 Iwasaki, A. & Medzhitov, R. Regulation of adaptive immunity by the innate immune system. *science* **327**, 291-295 (2010).

137 Coffman, R. L., Sher, A. & Seder, R. A. Vaccine adjuvants: putting innate immunity to work. *Immunity* **33**, 492-503 (2010).

138 Merlo, L. M. & Mandik-Nayak, L. in *Cancer Immunotherapy* 25-40 (Elsevier, 2013).

139 Broere, F. & van Eden, W. in *Nijkamp and Parnham's Principles of Immunopharmacology* 23-35 (Springer, 2019).

140 Constant, S. L. B lymphocytes as antigen-presenting cells for CD4+ T cell priming in vivo. *The Journal of Immunology* **162**, 5695-5703 (1999).

141 Ni, K. & O'neill, H. The role of dendritic cells in T cell activation. *Immunology and cell biology* **75**, 223-230 (1997).

142 Lee, S. & Nguyen, M. T. Recent advances of vaccine adjuvants for infectious diseases. *Immune network* **15**, 51-57 (2015).

143 Palucka, K., Banchereau, J. & Mellman, I. Designing vaccines based on biology of human dendritic cell subsets. *Immunity* **33**, 464-478 (2010).

144 Palm, N. W. & Medzhitov, R. Pattern recognition receptors and control of adaptive immunity. *Immunological reviews* **227**, 221-233 (2009).

145 Kapsenberg, M. L. Dendritic-cell control of pathogen-driven T-cell polarization. *Nature Reviews Immunology* **3**, 984-993 (2003).

146 Swain, S. L. CD4 T cell development and cytokine polarization: an overview. *Journal of leukocyte biology* **57**, 795-798 (1995).

147 Tarlinton, D. & Good-Jacobson, K. Diversity among memory B cells: origin, consequences, and utility. *Science* **341**, 1205-1211 (2013).

148 King, L. B. & Monroe, J. Immunobiology of the immature B cell: plasticity in the B-cell antigen receptor-induced response fine tunes negative selection. *Immunological reviews* **176**, 86-104 (2000).

149 Reif, K. *et al.* Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* **416**, 94-99 (2002).

150 MacLennan, I. C. *et al.* Extrafollicular antibody responses. *Immunological reviews* **194**, 8-18 (2003).

151 De Silva, N. S. & Klein, U. Dynamics of B cells in germinal centres. *Nature reviews immunology* **15**, 137-148 (2015).

152 MacLennan, I. C. Germinal centers. *Annual review of immunology* **12**, 117-139 (1994).

153 Linterman, M. A. & Vinuesa, C. G. T follicular helper cells during immunity and tolerance. *Progress in molecular biology and translational science* **92**, 207-248 (2010).

154 Crotty, S. Follicular helper CD4 T cells (Tfh). *Annual review of immunology* **29**, 621-663 (2011).

155 Rasheed, M. A. U. *et al.* Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. *Journal of virology* **87**, 7737-7746 (2013).

156 Zehentmeier, S. *et al.* Static and dynamic components synergize to form a stable survival niche for bone marrow plasma cells. *European journal of immunology* **44**, 2306-2317 (2014).

157 Amanna, I. J. & Slifka, M. K. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. *Immunological reviews* **236**, 125-138 (2010).

158 Kurosaki, T., Kometani, K. & Ise, W. Memory B cells. *Nature Reviews Immunology* **15**, 149-159 (2015).

159 Senne, D., King, D. & Kapczynskiº, D. by Vaccination. *Dev Biol. Basel* **119**, 165-170 (2004).

160 Dortmans, J. C., Peeters, B. P. & Koch, G. Newcastle disease virus outbreaks: vaccine mismatch or inadequate application? *Veterinary microbiology* **160**, 17-22 (2012).

161 Nikbakht Brujeni, G., Hassanzadeh, M., Al-Karagoly, H., Tolouei, T. & Esmailnejad, A. Evaluation of humoral immune responses to enterotropic lentogenic VG/GA vaccine of Newcastle disease in commercial turkey poults (Meleagris gallopavo). *Acta Veterinaria Scandinavica* **61**, 1-6 (2019).

162 Reynolds, D. & Maraqa, A. Protective immunity against Newcastle disease: the role of cell-mediated immunity. *Avian diseases*, 145-154 (2000).

163 Lambrecht, B., Gonze, M., Meulemans, G. & van denberg, T. P. Assessment of the cell-mediated immune response in chickens by detection of chicken interferon-γ in response to mitogen and recall Newcastle disease viral antigen stimulation. *Avian Pathology* **33**, 343-350 (2004).

164 Al-Garib, S., Gielkens, A. L. J., Gruys, E., Hartog, L. & Koch, G. Immunoglobulin class distribution of systemic and mucosal antibody responses to Newcastle disease in chickens. *Avian diseases* **47**, 32-40 (2003).

165 Sedeik, M. *et al.* Comparative efficacy of commercial inactivated Newcastle disease virus vaccines against Newcastle disease virus genotype VII in broiler chickens. *Poultry science* **98**, 2000-2007 (2019).

166 Piétu, G. *et al.* The Genexpress IMAGE knowledge base of the human brain transcriptome: a prototype integrated resource for functional and computational genomics. *Genome research* **9**, 195-209 (1999).

167 Bainbridge, M. N. *et al.* Analysis of the prostate cancer cell line LNCaP transcriptome using a sequencing-by-synthesis approach. *BMC genomics* **7**, 1-11 (2006).

168 Wilhelm, B. T. *et al.* Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. *Nature* **453**, 1239-1243 (2008).

169 Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature methods* **5**, 621-628 (2008).

170 Ozsolak, F. & Milos, P. M. RNA sequencing: advances, challenges and opportunities. *Nature reviews genetics* **12**, 87-98 (2011).

171 Lowe, R., Shirley, N., Bleackley, M., Dolan, S. & Shafee, T. Transcriptomics technologies. *PLoS computational biology* **13**, e1005457 (2017).

172 Van Verk, M. C., Hickman, R., Pieterse, C. M. & Van Wees, S. C. RNA-Seq: revelation of the messengers. *Trends in plant science* **18**, 175-179 (2013).

173 Andrews, S. (Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom, 2010).

174 Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature biotechnology* **37**, 907-915 (2019).

175 Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923-930 (2014).

176 Liu, L. *et al.* Comparison of next-generation sequencing systems. *Journal of Biomedicine and Biotechnology* **2012** (2012).

177 Quail, M. A. *et al.* A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC genomics* **13**, 1-13 (2012).

178 Ryu, W.-S. *Molecular virology of human pathogenic viruses*. (Academic Press, 2016).

179 Grimes, S. E. *A Basic Laboratory Manual for the Small-Scale Production and Testing of I-2 Newcastle Disease Vaccine*. (FAO, 2002).

180 Bourgeois, M. A. & Oaks, J. L. in *Equine Infectious Diseases* 132-140. e132 (Elsevier, 2014).

181 Singh, R. S., Singh, T. & Singh, A. K. in *Advances in enzyme technology* 225-271 (Elsevier, 2019).

182 Gan, S. D. & Patel, K. R. Enzyme immunoassay and enzyme-linked immunosorbent assay. *J Invest Dermatol* **133**, e12 (2013).

183 Butler, J. E. Enzyme-linked immunosorbent assay. *Journal of immunoassay* **21**, 165-209 (2000).

184 Paulie, S., Perlmann, P. & Perlmann, H. in *Cell Biology* 533-538 (Elsevier, 2006).

185 Hosseini, S., Vázquez-Villegas, P., Rito-Palomares, M. & Martinez-Chapa, S. O. in *Enzyme-linked immunosorbent assay (ELISA)* 31-56 (Springer, 2018).

186 Yang, M. *et al.* Development and application of monoclonal antibodies against avian influenza virus nucleoprotein. *Journal of virological methods* **147**, 265-274 (2008).

187 Chambers, P., Millar, N. S. & Emmerson, P. T. Nucleotide sequence of the gene encoding the fusion glycoprotein of Newcastle disease virus. *Journal of general virology* **67**, 2685-2694 (1986).

188 McGinnes, L. & Morrison, T. Nucleotide sequence of the gene encoding the Newcastle disease virus fusion protein and comparisons of paramyxovirus fusion protein sequences. *Virus research* **5**, 343-356 (1986).

189 Chen, L. *et al.* The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. *Structure* **9**, 255-266 (2001).

190 de Leeuw, O. S., Hartog, L., Koch, G. & Peeters, B. P. Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus: non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. *Journal of General Virology* **84**, 475-484 (2003).

191 Griffin, D. E., Howley, P. M. & Knipe, D. M. *Fields' virology*. (Lippincott Williams & Wilkins, 2007).

192 Tan, S. W., Ideris, A., Omar, A. R., Yusoff, K. & Hair-Bejo, M. Sequence and phylogenetic analysis of Newcastle disease virus genotypes isolated in Malaysia between 2004 and 2005. *Archives of virology* **155**, 63-70 (2010).

193 Fujii, Y., Sakaguchi, T., Kiyotani, K. & Yoshida, T. Comparison of substrate specificities against the fusion glycoprotein of virulent Newcastle disease virus between a chick embryo fibroblast processing protease and mammalian subtilisin-like proteases. *Microbiology and immunology* **43**, 133-140 (1999).

194 Nagai, Y., Klenk, H.-D. & Rott, R. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. *Virology* **72**, 494-508 (1976).

195 Panda, A., Huang, Z., Elankumaran, S., Rockemann, D. D. & Samal, S. K. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microbial pathogenesis* **36**, 1-10 (2004).

196 Samal, S., Kumar, S., Khattar, S. K. & Samal, S. K. A single amino acid change, Q114R, in the cleavage-site sequence of Newcastle disease virus fusion protein attenuates viral replication and pathogenicity. *Journal of general virology* **92**, 2333-2338 (2011).

197 Le, L., Brasseur, R., Wemers, C., Meulemans, G. & Burny, A. Fusion (F) protein gene of Newcastle disease virus: sequence and hydrophobicity comparative analysis between virulent and avirulent strains. *Virus Genes* **1**, 333-350 (1988).

198 Yusoff, K. *et al.* Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain Beaudette C. *Journal of general virology* **70**, 3105-3109 (1989).

199 Toyoda, T., Gotoh, B., Sakaguchi, T., Kida, H. & Nagai, Y. Identification of amino acids relevant to three antigenic determinants on the fusion protein of Newcastle disease virus that are involved in fusion inhibition and neutralization. *Journal of virology* **62**, 4427-4430 (1988).

200 Abenes, G., Kida, H. & Yanagawa, R. Antigenic mapping and functional analysis of the F protein of Newcastle disease virus using monoclonal antibodies. *Archives of virology* **90**, 97-110 (1986).

201 Neyt, C. *et al.* Mutations located on both F1 and F2 subunits of the Newcastle disease virus fusion protein confer resistance to neutralization with monoclonal antibodies. *Journal of virology* **63**, 952-954 (1989).

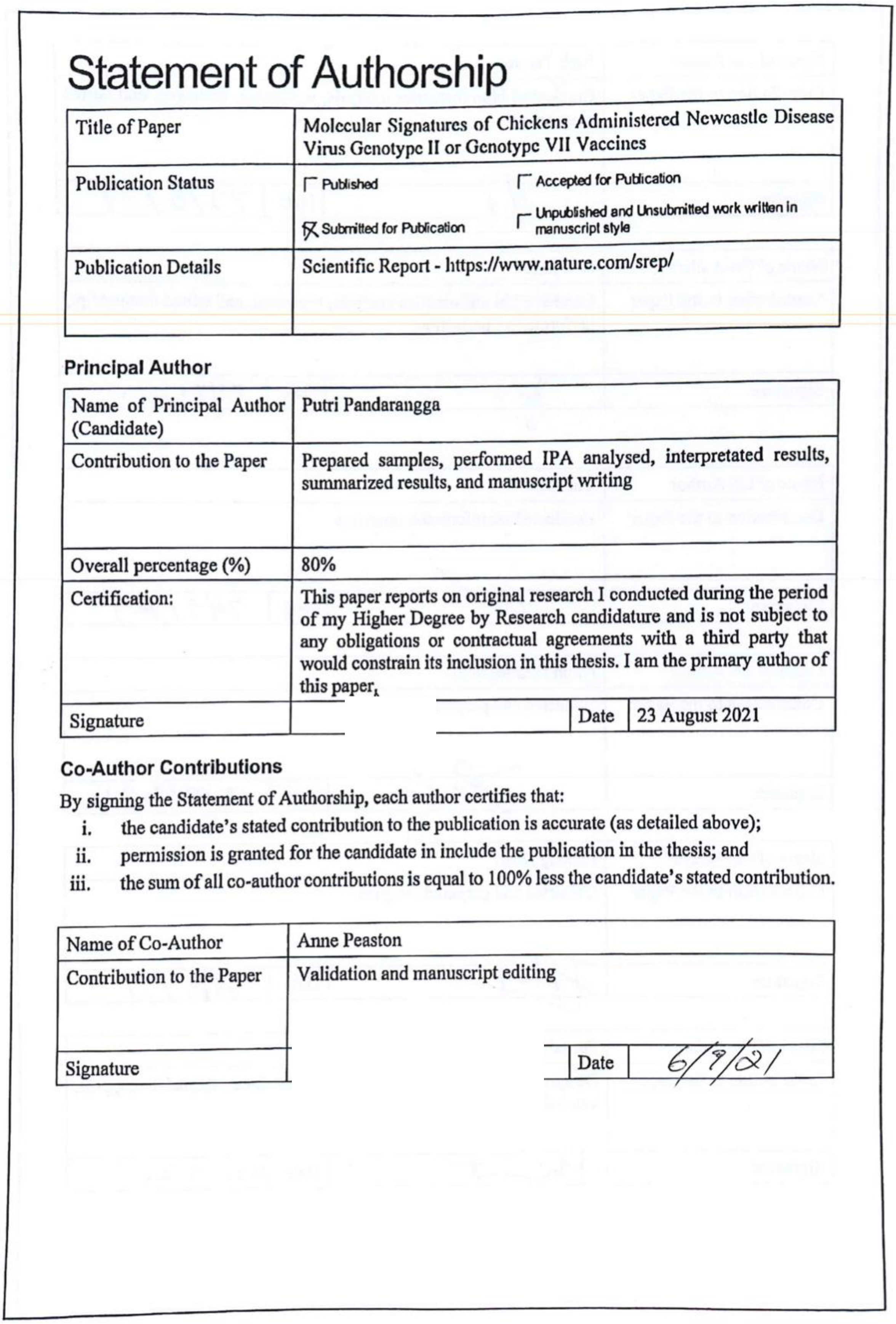
202 Choi, K.-S., Lee, E.-K., Jeon, W.-J. & Kwon, J.-H. Antigenic and immunogenic investigation of the virulence motif of the Newcastle disease virus fusion protein. *Journal of veterinary science* **11**, 205-211 (2010).

203 Haworth, A., Savage, H. & Lench, N. Diagnostic Genomics and Clinical Bioinformatics. *Medical and Health Genomics*, 37-50 (2016).

204 Doan, P. T. K. *et al.* Genome Sequences of Newcastle Disease Virus Strains from Two Outbreaks in Indonesia. *Microbiology Resource Announcements* **9** (2020).

CHAPTER 2

MOLECULAR SIGNATURES OF CHICKENS ADMINISTERED NEWCASTLE DISEASE VIRUS GENOTYPE II OR GENOTYPE VII VACCINES



Statement of Authorship

Title of Paper	Molecular Signatures of Chickens Administered Newcastle Disease Virus Genotype II or Genotype VII Vaccines
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Scientific Report - https://www.nature.com/srep/

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Name of Principal Author (Candidate)	Putri Pandarangga		
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Overall percentage (%)	80%		
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.		
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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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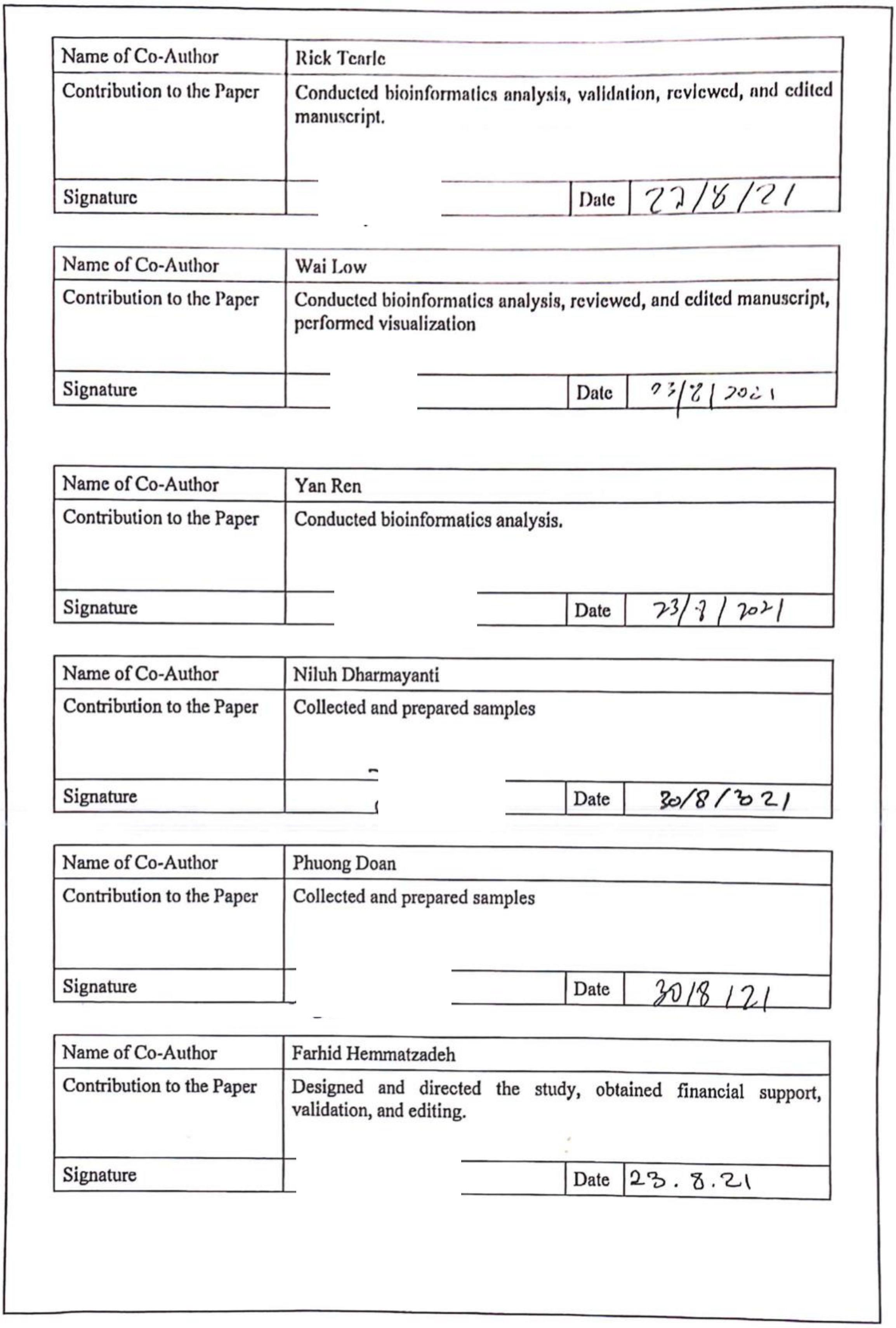

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Molecular Signatures of Chickens Administered Newcastle Disease Virus Genotype II or Genotype VII Vaccines

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The manuscript is under review in the **Scientific Report** Journal. However, the layout of figures and tables are embedded in the text are different from the journal.

Abstract

Newcastle Disease Virus (NDV) genotype VII (GVII) causes death in chickens in the field even if they are vaccinated with a common NDV genotype II vaccine (GIIvacc). To overcome this, the GVII strain has been used as a vaccine to prevent NDV outbreaks. However, the debate about vaccine differences in terms of serology and viral shedding terms remains ongoing. Hence, this study investigated the difference in chicken responses to the two vaccines at the molecular level. The transcriptomes of spleens from vaccinated chickens predicted that GVIIvacc significantly affected immune response by downregulating neuroinflammation and enhancing other responses such as the synaptogenesis pathway in the nervous system. Furthermore, neuron activities on spleen transcriptomic showed that GVIIvacc had a neurotropic characteristic. We speculated simultaneously that the down-regulated immune system regulation correlated with protecting the nervous system from excess leukocytes and cytokine activity. In contrast, the GIIvacc was predicted to inhibit apoptosis by downregulating PERK/ATF4/CHOP as part of the unfolded protein response (UPR) pathway and did not affect the nervous system. Thus, the application of GVIIvacc needs to be considered in countries where GVII is the leading cause of NDV outbreaks. The predicted molecular signatures may also be used in developing new vaccines that trigger specific genes in the immune system and can provide markers to test a vaccine's efficacy in combating NDV outbreaks.

Keywords: NDV; transcriptomic; vaccine; NDV genotype II; NDV genotype VII, spleen

2. 1. Introduction

Newcastle Disease (ND) is caused by the virulent Newcastle Disease Virus (vNDV), a major transboundary animal disease of chickens that engenders large economic losses annually [1]. This virus belongs to the genus *Orthoavulavirus* in the family *Paramyxoviridae* [2] and has caused five panzootic events since 1926 [3]. In the latest phylogenetic classification of avian paramyxoviruses, NDV class II contains twenty-one genotypes that are either virulent or non-virulent [4]. The most virulent NDV strains, causing outbreaks worldwide, including Africa, Asia, and Eastern Europe, belong to genotype VII (GVII) [5-8]. However, the commercial vaccines in use since the 1950s are derived from genotypes I (GI) and II (GII) [9]. GII and GVII are substantially different, with 21.6% of nucleotides discordant, where 10% is the threshold to establish a new genotype [4,10].

The antigenic difference between the NDV vaccine and NDV circulating in the field is likely one of the main reasons that ND outbreaks continue. In Indonesia, the mortality of GII-vaccinated chickens reaches 70-80%, which has been proposed due to antigenic differences that affect the ability of the commercial vaccine to provide protection [11]. Consequently, a vaccine has been derived from GVII (GVIIvacc) [12-14] to replace the standard GII vaccine (GIIvacc). However, antigenic differences as a reason for vaccine failure are still a topic of concern amongst NDV researchers. An NDV vaccine made from circulating NDV, known as a homologous vaccine, can protect chickens better in terms of reducing the viral load [13,15]. Despite this, Cornax *et al*. (2012) hypothesized that the GIIvacc known as LaSota vaccine can still protect chickens from GVII as long as it is applied correctly, and no maternal antibodies are present [16]. Moreover, the commercial vaccine can still reduce viral shedding after the challenge with virulent NDV [17]. However, these studies only considered serology, clinical signs, and viral load count, limiting utility in considering a vaccine’s applicability. In addition, chickens vaccinated with either GIIvacc or GVIIvacc still need to be investigated to understand the host response at the gene level.

Unlike the situation with human vaccines, only a few studies of chicken vaccines have utilized transcriptomics to identify molecular signatures in vaccinated chickens [18-20]. Analysis of the host transcriptional response to human vaccines has identified differentially expressed genes that mark specific immune responses [21,22]. This approach involves biology, microbiology, bioinformatics, computational modeling, statistical analysis, and computer science. Since 2010, it has been used to predict vaccine-induced human immune responses [23]. Every vaccine has its molecular signature when stimulating an immune response. For instance, in humans, interferon signaling pathways involving genes controlling STAT1, STAT2, TLR7, IRF3, and IRF7 were identified three days after vaccination with Live Attenuated Influenza Virus (LAIV) [24]. In comparison, Yellow Fever-17D (YF-17D) induced the activity of TLR2, TLR7, TLR8, and TLR9 in human dendritic cells to stimulate an innate and adaptive immune response [25].

The mechanisms whereby non-virulent and virulent NDV vaccines stimulate the chicken immune system remain obscure. Furthermore, molecular signatures, consisting of pathways and genes that protect the chicken, have not been thoroughly investigated for GIIvacc and GVIIvacc. Hence, this study aimed to identify the significant pathways and genes in chicken after the second injection of the GIIvacc or GVIIvacc to identify molecular signatures for each. Moreover, based on IPA prediction, we wanted to know the difference between host response, including immune regulation, to the commercial vaccine and GVIIvacc as a homologous vaccine at the molecular level. This knowledge may lead to new insights into developing potent vaccines by targeting the specific genes that stimulate transcription factors related to chicken immune response. In addition, the predicted molecular signatures could be applied as biomarkers to differentiate sera from vaccinated and natural infections.

2. 2. Results

2. 2. 1. Summary of RNA-Sequencing Data

Counts of RNA-seq reads from chicken splenic RNA across the three groups are summarized in Table 2. 1. Up to 16% of raw reads were removed during filtering, an acceptable proportion [26]. Thus, up to 89% of cleaned reads mapped to the chicken genome (GRCg6a). A total of 15,355 genes were expressed across all of the groups.

Table 2. 1. The summary of spleen RNA-seq reads alignment to galGal6a.

Categories	**Control**	**GII Vaccine**	**GVII vaccine**
Total raw reads	61,374,003	85,732,634	155,252,379
Total clean reads	55,901,725	80,348,805	140,494,040
Total mapped	88.26%	88.51%	89.25%

2. 2. 2. Validation of RNA-Sequencing Results Using qPCR

Quantitative PCR was used to validate the RNA-seq results. The correlation between qPCR and RNA sequencing data was high (R = 0.83). Amplification efficiency (AE) of all selected genes ranges from 95% to 109% (Table 2. 2).

Table 2. 2. Primers for Validation of RNA-seq Data with qPCR

NCBI Accession Number	Gene Name	Primer		Ta (°C)	AE (%)	Size (bp)
		Forward	Reverse			
NM_205424.1	ANOS1	CCAAAGCTTCTGTGAGCCTCT	TGGGAACTTGGCATGTGTGA	60	98.5	224
NM 001198752.1	CCR7	GACCATGGACGGCGGTAAAC	CGGTGACGTTGTTCCCAGCA	60	107.17	89
NM_001277996.1	IL16	GCTTCAGTCTGGAAGGTGG	TGTTCCAACGAGGTCCCTTT	58	97	88
XM_416914.6	IL1R2	AGGATGCAGAACCACAGATTTCA	CAGGTTCTCCGTGCAGTTCA	60	108.7	205
XM_015293006.2	OASL	GGAGTCAGCATCACCAGTCC	CTGAATCACCTGCCCCAGTG	64	109	144
NM_001039097.1	SLCO1C1	CCAGTGCACTCAGATACGTG	CCGAAGAACCCACAGGACAG	60	99.8	92
NC_006088.5	RAC2	AGGATTACGACAGGCTGAGGC	GATGCTGGGCTGACAAGGGA	61	110	82
AB495656.1	ACTB	CCAGACATCAGGGTGTGATGG	CTCCATATCATCCCAGTTGGTGA	60	95	137
NC_006088.5	GAPDH	GAAGGCTGGGGCTCATCTG	CAGTTGGTGGTGCACGATG	60	104.93	150

2. 2. 3. Results of Vaccinated Chickens DEGs

When a False Discovery Rate (FDR) of ≤ 0.05 and log fold change of ±1 were set as thresholds, only 89 genes from GIIvacc vs. control and 2751 genes from GVIIvacc vs. control were reported as DEGs. Using 2 as an absolute z-score, 12 significantly differentially expressed pathways were identified for GIIvacc vs. control (Figure 2. 1) and 47 for GVIIvacc vs. control (Figure 2. 2). Each pathway contained up-and down-regulated genes (Table 2. 3).

Table 2. 3. The most up-and down-regulated genes from canonical pathways correlated with chickens' immune response

Categories	**Up-regulated genes**	**Down-regulated genes**	**P-value**
a) Significant Pathways of DEGs GIIvacc			
Unfolded Protein Response (UPR)	N/A	CEBPB, HSPA (5/8), HSPH (1/2)	1.78E-06
CXCR4 signaling	N/A	CXCR4, EGR1, FOS, MYL (4/9)	3.48E-04
IL-6 signaling	N/A	CEBPB, FOS, HSPB (1/7)	1.09E-03
Leukocyte extravasation	N/A	ACTA1, ACTG2, CXCR4, THY1	5.65E-03
b) Significant Pathways of DEGs GVIIvacc			
Neuroinflammation signaling pathway	ACVR2B, AKT3, BDNF, CREB5, CALB1, GABRA (1/2/3/4/6), GABRB (1/2/3), GABBR2, GABRG (1/2), GRIA1, GRIN (1/2A/2B), KCNJ6, NTF3, NOX3, PIK3C2G, PLA2 (G4B/G4E/G4F/G10), SNCA, SLC (1A3/6A1/6A11), TGFB2	CCL5, CYBB, CX3CR1, CRP, DRA, FOS, HLA-A, HLA-IL1R1, JAK1, P2RX7, PIK3R5, TREM2, TLR (2/7)	1.11E-07
Leukocyte extravasation signaling	ACTN2, AFDN, BMX, CDH5, CLDN (1/10), CTNNA (2/)3, DLC1, EDIL3, MMP16, NOX3, PIK3C2G, PRKCE, TIMP4	ACTA1, ACTG2, CYBB, ITGB3, MMP9, PIK3R5, PRKCH, PTK2B, RHOH, RAC2, THY1	1.57E-07
IL-15 production	BMX, EGFR, EPH (A3/A5/B2), ERBB4, FLT1, FRK, MET, MUSK, RET, ROR2, ROS1, STYK1, YES1	BTK, CSK, MATK, PTK2B, ZAP70	3.63E-06
PKCθ signaling in T lymphocyte	CACNA (1B/1C/1D/1G/1I/2D1/2D2/2D3), CACN (G2/B2), MAP3K15, PIK3C2G	CD (3D/3E/4/28/247), CACNB4, FOS, GRAP2, HLA(A/DRA), LCP2, NFKBIE, PIK3R5, RAC2, ZAP70	4.35E-06
iCOS-iCOSL signaling in T helper cells	AKT3, CAMK4, GAB2, PIK3C2G	CD (3D/3E/4/28/40L/247), CSK, GRAP2, HLA(A/DRA), INPP5D, IL2RG, LCP2, NFKBIE, PLEKHA2, PTPRC, PIK3R5, TRAT1, ZAP70	1.20E-05
IL-9 signaling	PIK3C2G, SOCS2	IL2RG, JAK1, PIK3R5	2.65E-04
Role of NFAT in the regulation of the immune response	AKT3, CAMK4, GNAS, PIK3C2G, PLCB (1/4)	BTK, CD(3D/3E/4/28/79B/247), FOS, HLA (A/DRA), LCP2, NFKBIE, PIK3R5, ZAP70	7.49E-04
Synaptogenesis signaling pathway	ADCY (1/2/8), AKT3, BDNF, CACNA1B, CAMK4, CNTNAP2, CDH (4/6/7/8/9/10/12/13/18/19/20), CPLX1, CREB5, EPHA (3/5/7), EFNA5, GRIA (1/2/4), GRM (1/5/7/8), GRIN1, GRIN2 (A/B), NLGN (1/4Y), Nrxn3, NRXN1, PIK3C2G, PRKAR2B, SYT (1/4/9/14/17), SNCA, STXBP6, THBS2, YES1	ADCY7, ARPC1B, CACNB4, CDH3, SYT (2/8)	1.23E-20

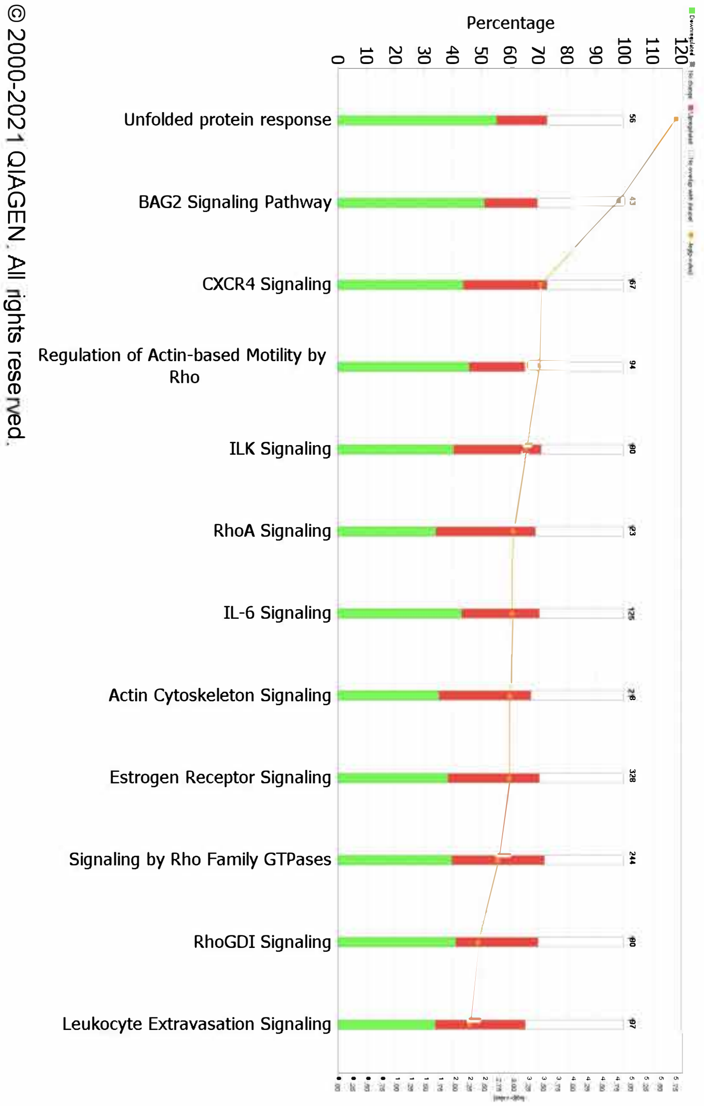


Figure 2. 1. Twelve significant pathways from GIIvacc DEGs. DEGs were selected with a-log (p-value) >2 and an absolute z-score was >2 as a cutoff. The number on the top of each bar represents the total number of genes contributing to the pathways. Each bar has three colors: green, red, and white, represented as down-regulated, up-regulated, and no overlap genes with the basic knowledge in IPA. The –log (p-value) of each pathway is indicated by the orange line.

B

Figure 2. 2. Forty-seven pathways from GVIIvacc DEGs. DEGs were selected with a-log (p-value) >2 and an absolute z-score was >2 as a cutoff. The number on the top of each bar represents the total number of genes contributing to the pathways. Each bar has three colors: green, red, and white, represented as down-regulated, up-regulated, and no overlap genes with the basic knowledge in IPA. The –log (p-value) of each pathway is indicated by the orange line.

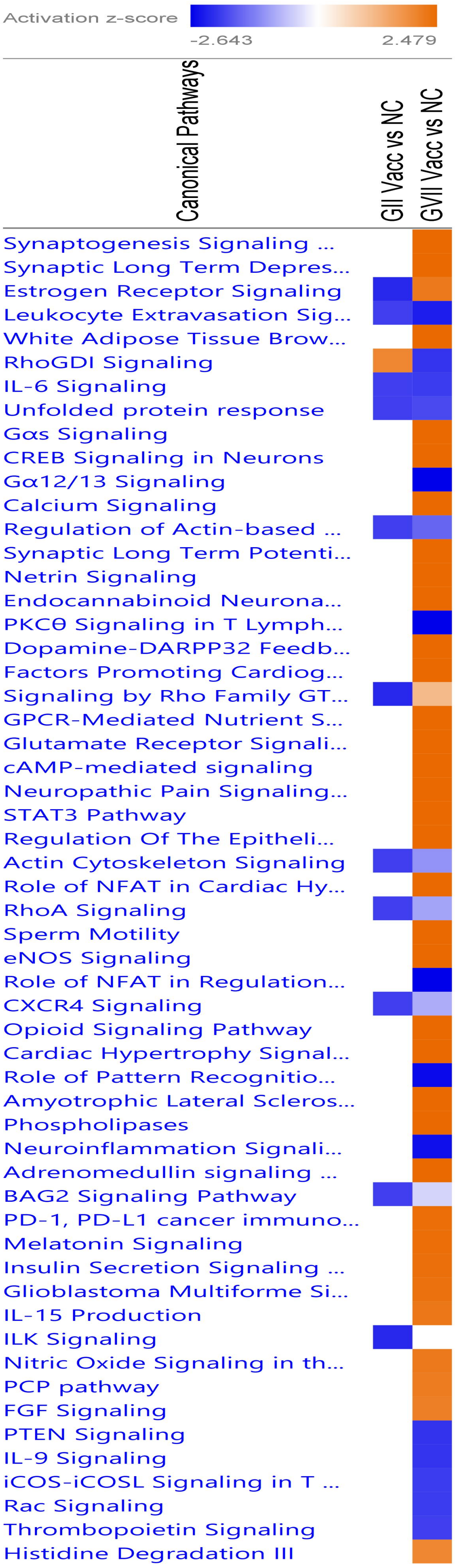


Figure 2. 3. Heatmap. Comparing GIIvacc and GVIIvacc DEGs shows orange boxes as activated pathways, blue boxes as inhibited pathways, and white boxes as non-significant pathways. These pathways are filtered with a –log (P-value) >2.5 and z-score >2. The contrast in the heatmap was visualized with a z-score feature.

2. 2. 4. Pathways and Genes Altered in Chickens Vaccinated with GIIvacc

Chickens that were vaccinated with GIIvacc showed 12 differentially regulated pathways (Figure 2. 1). These pathways fell into two main groups, immune response regulation and essential cellular functions. The immune response regulation group contained cytokine signaling genes (CXCR4 signaling and IL-6) and cellular immune response genes (leukocyte extravasation genes). All the pathways in this group were down-regulated. The basic cellular function group contained: 1) cellular stress and injury genes, 2) cellular growth, proliferation, and development genes, 3) intracellular and second messenger genes. Similar to the immune response regulation group, all the pathways in these groups were down-regulated except for the RhoGDI signaling pathway (Figure 2. 3). In addition, some genes that have a role in controlling immune response were down-regulated such as CEBPB, CXCR4, and THY1 (Table 2. 3).

The Unfolded Protein Response (UPR) pathway (Figure 2. 4) was a part of the cellular stress and injury group. This pathway regulated responders to Endoplasmic Reticulum (ER) stress, including stress transducers as PERK, IRE1, and ATF6. All these transducers were down-regulated (blue color in Figure 2. 4). PERK is a part of EIF2α and was down-regulated, suppressing the protein that causes apoptosis. XBP-1, a gene downstream of IRE1, was down-regulated. In addition, actin cytoskeleton signaling, regulation of actin-based motility by Rho, and ILK signaling involved in cellular growth, proliferation, and development were down-regulated (not depicted). As part of the intracellular and second messenger group, GTPase signaling family genes such as Rac, Rho, and cdc42 played a dominant role in cellular development pathways in chickens vaccinated with GIIvacc. Moreover, the activities of the family GTPase as the second messenger were down-regulated in the leukocyte extravasation pathway (Figure 2. 5).

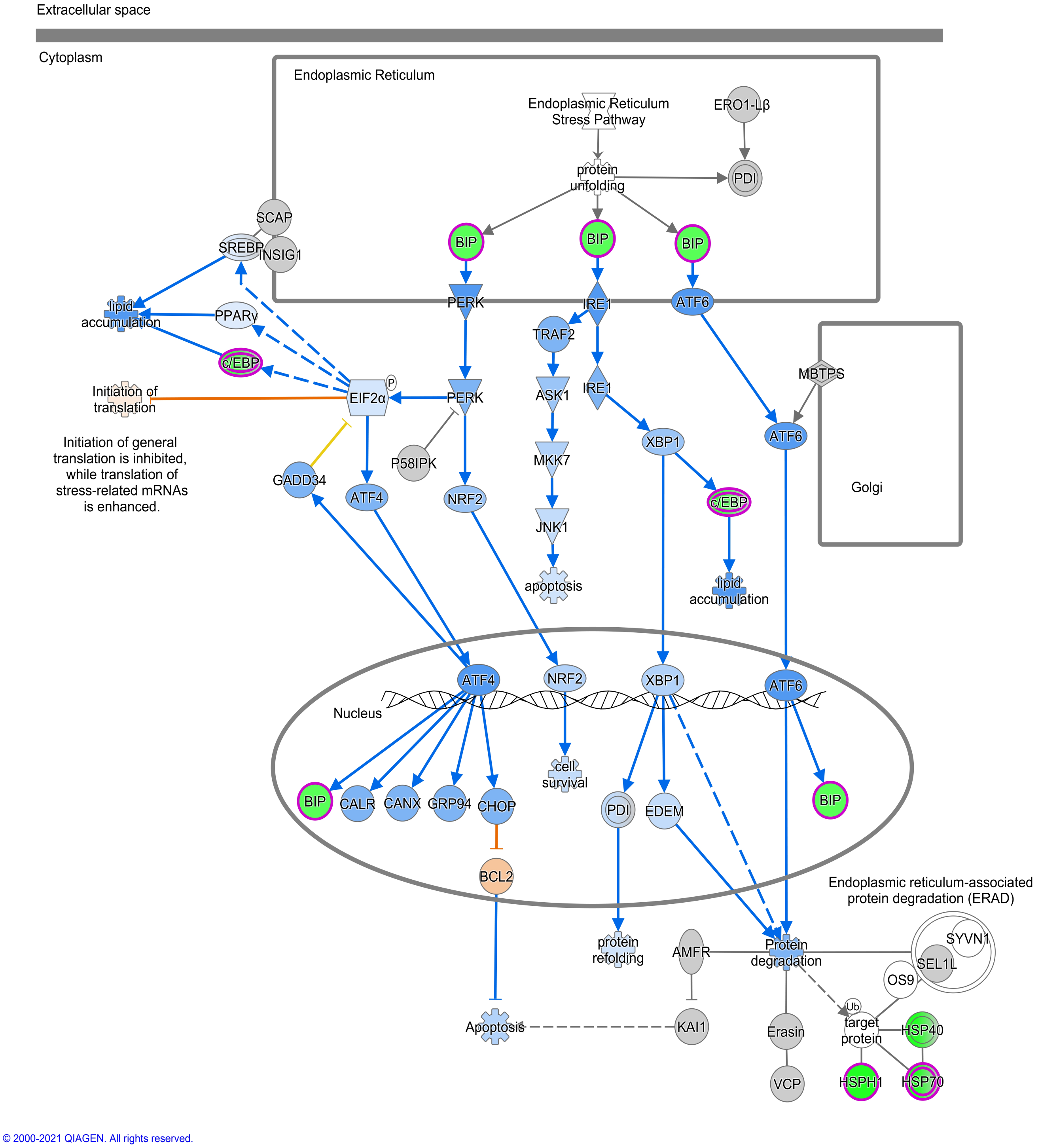
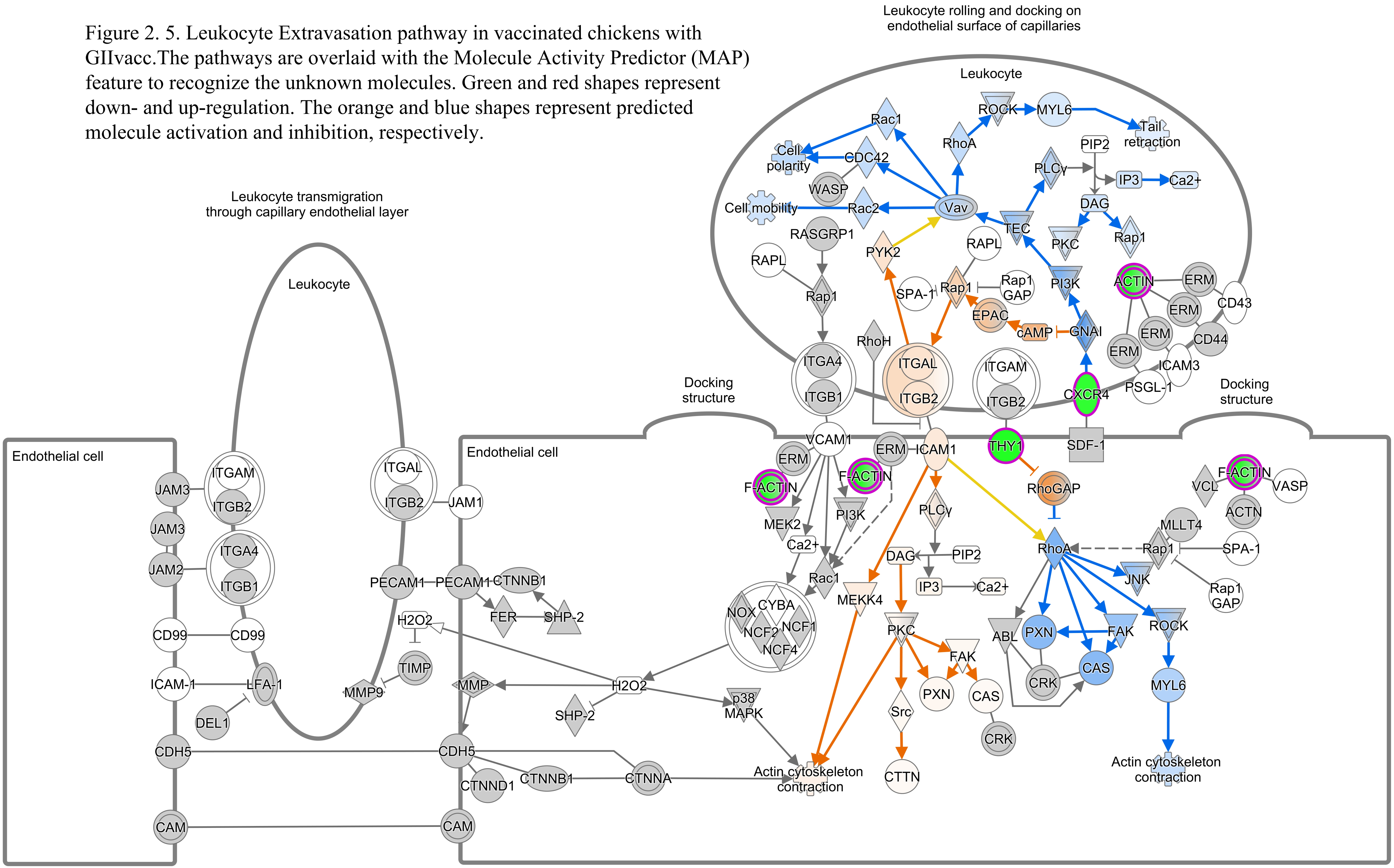


Figure 2. 4. Unfolded Protein Response (UPR) pathway in vaccinated chickens with GIIvacc.
The pathways are overlaid with the Molecule Activity Predictor (MAP) feature to recognize the unknown molecules. Green and red shapes represent down- and up-regulation. The orange and blue shapes represent predicted molecule activation and inhibition, respectively.

Figure 2. 5. Leukocyte Extravasation pathway in vaccinated chickens with GIIvacc.The pathways are overlaid with the Molecule Activity Predictor (MAP) feature to recognize the unknown molecules. Green and red shapes represent down- and up-regulation. The orange and blue shapes represent predicted molecule activation and inhibition, respectively.



2. 2. 5. Genes and Pathways Altered in Chickens Vaccinated with GVIIvacc

Forty-seven pathways were differentially regulated in the GVIIvacc vs. control (Figure 2. 2). These pathways were grouped into 1) immune system regulation consisting of cellular immune response (neuroinflammation signaling pathway, leukocyte extravasation, and regulation of T lymphocytes), cytokine signaling (IL-6 and IL-9 signaling), and humoral immune response (NFAT regulating the immune response); 2) essential cellular functions comprised UPR pathway as stress and injury cell response; 3) nervous system signaling contained synaptogenesis signaling, Long Term Depression (LTD), and Long term Potentiation (LTP) signaling.

Pathways correlated with immune system regulation were down-regulated, including the neuroinflammation signaling pathway (Figure 2. 6), leukocyte extravasation (Figure 2. 7), and NFAT regulating immune response (Figure 2. 8). NF-ƙB (marked with green circles) in microglial cells was a part of neuroinflammation signaling (Figure 2. 6). The downregulated NF-ƙB is capable of inhibiting the activities of downstream proteins such as IAP, BCL-2, pro-inflammatory, anti-apoptotic proteins shown with blue squares. However, the downregulated NF-ƙB can also activate NTF3 in microglia cells, shown in a pink square. In the neuroinflammation pathway, IFN γ in astrocytes was down-regulated and prevent the activity of downstream proteins such as T cell, CD4+, CD8+ recruitments and microglia activation that showed with blue shapes (Figure 2. 6).

In contrast, the pathways involved in the nervous system signaling tended to be up-regulated, specifically the synaptogenesis signaling pathway as neuron communication (Figure 2. 9). This communication between neurons involves three regions: pre-synaptic on the tip of the axon, synaptic cleft, and post-synaptic neuron in the dendritic spine, as shown in Figure 2. 9). Glutamate as a neurotransmitter can activate the intracellular signaling pathways such as LTD, LTP, and cAMP pathways in the neuron reflected with orange shapes in this data set.

Altogether, they activated the cAMP-responsive element-binding protein (CREB) as a nuclear transcription factor that triggers gene expression. In addition, some proteins marked with orange colors, such as neuronal adhesion, synaptic spine density, microtubule, and synapse stabilization, were activated in this DEG list (Figure 2. 9).

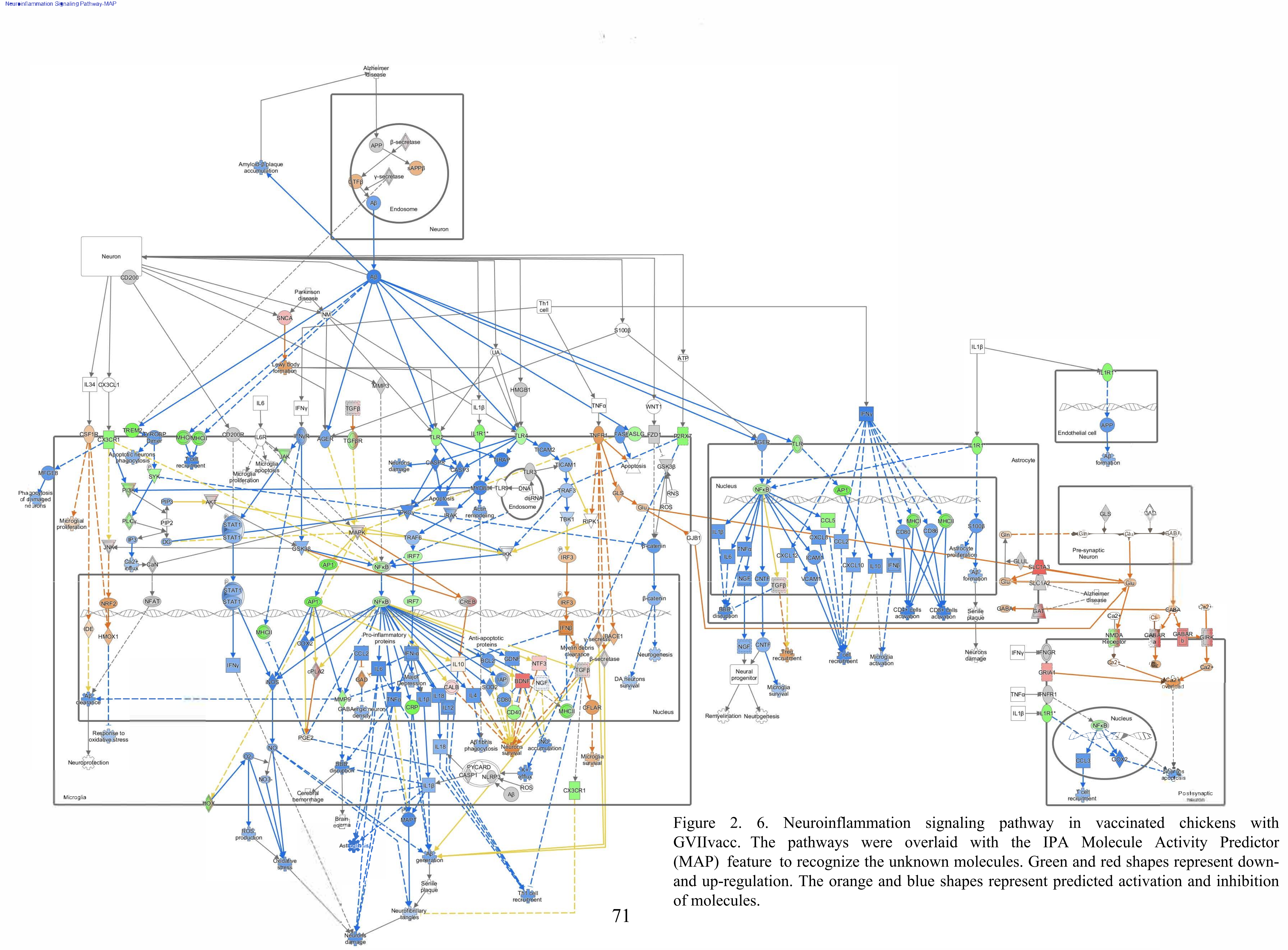
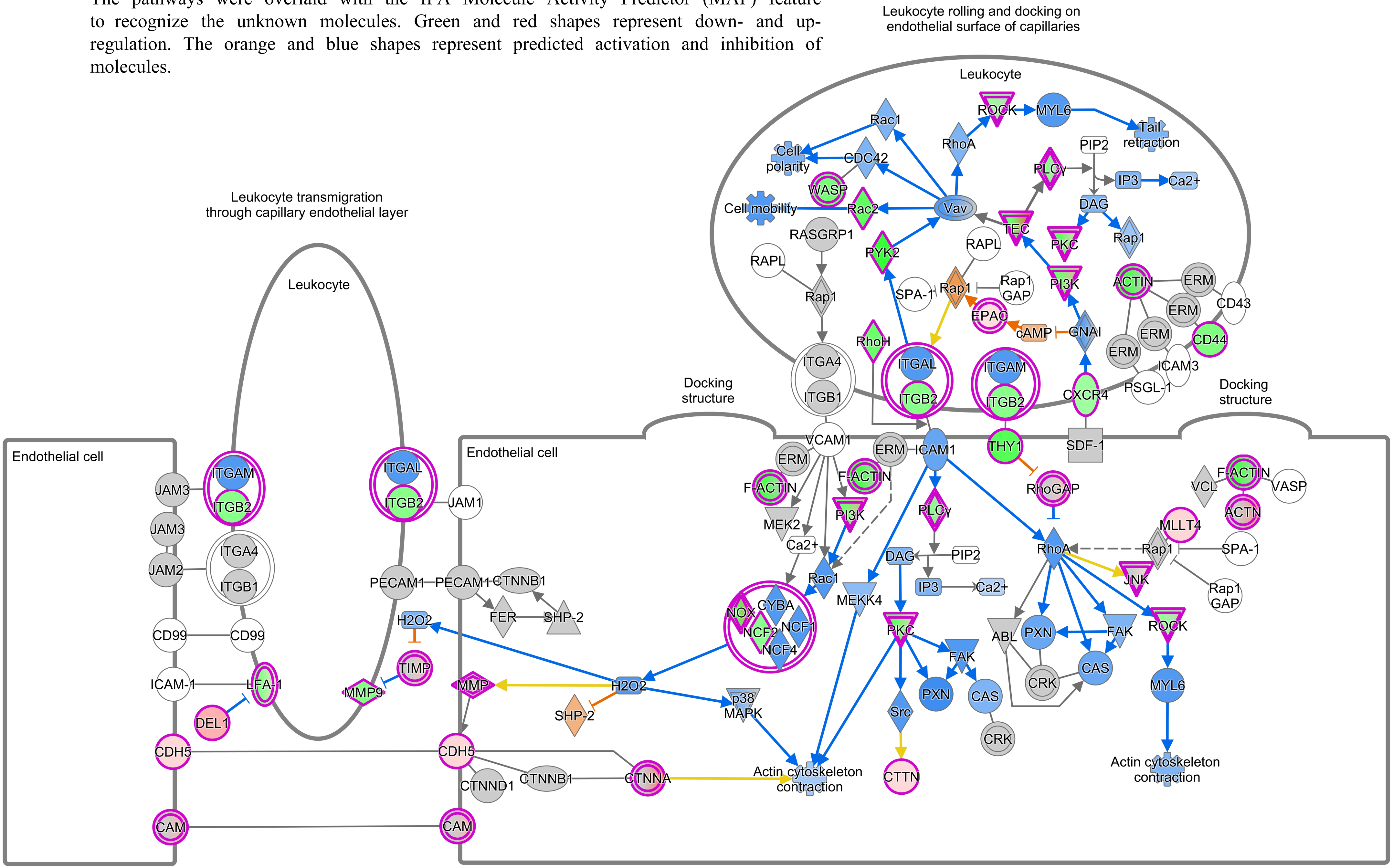


Figure 2. 6. Neuroinflammation signaling pathway in vaccinated chickens with GVIIvacc. The pathways were overlaid with the IPA Molecule Activity Predictor (MAP) feature to recognize the unknown molecules. Green and red shapes represent down- and up-regulation. The orange and blue shapes represent predicted activation and inhibition of molecules.

Figure 2. 7. Leukocyte extravasation signaling pathway in vaccinated chickens with GVIIvacc. The pathways were overlaid with the IPA Molecule Activity Predictor (MAP) feature to recognize the unknown molecules. Green and red shapes represent down- and up-regulation. The orange and blue shapes represent predicted activation and inhibition of molecules.



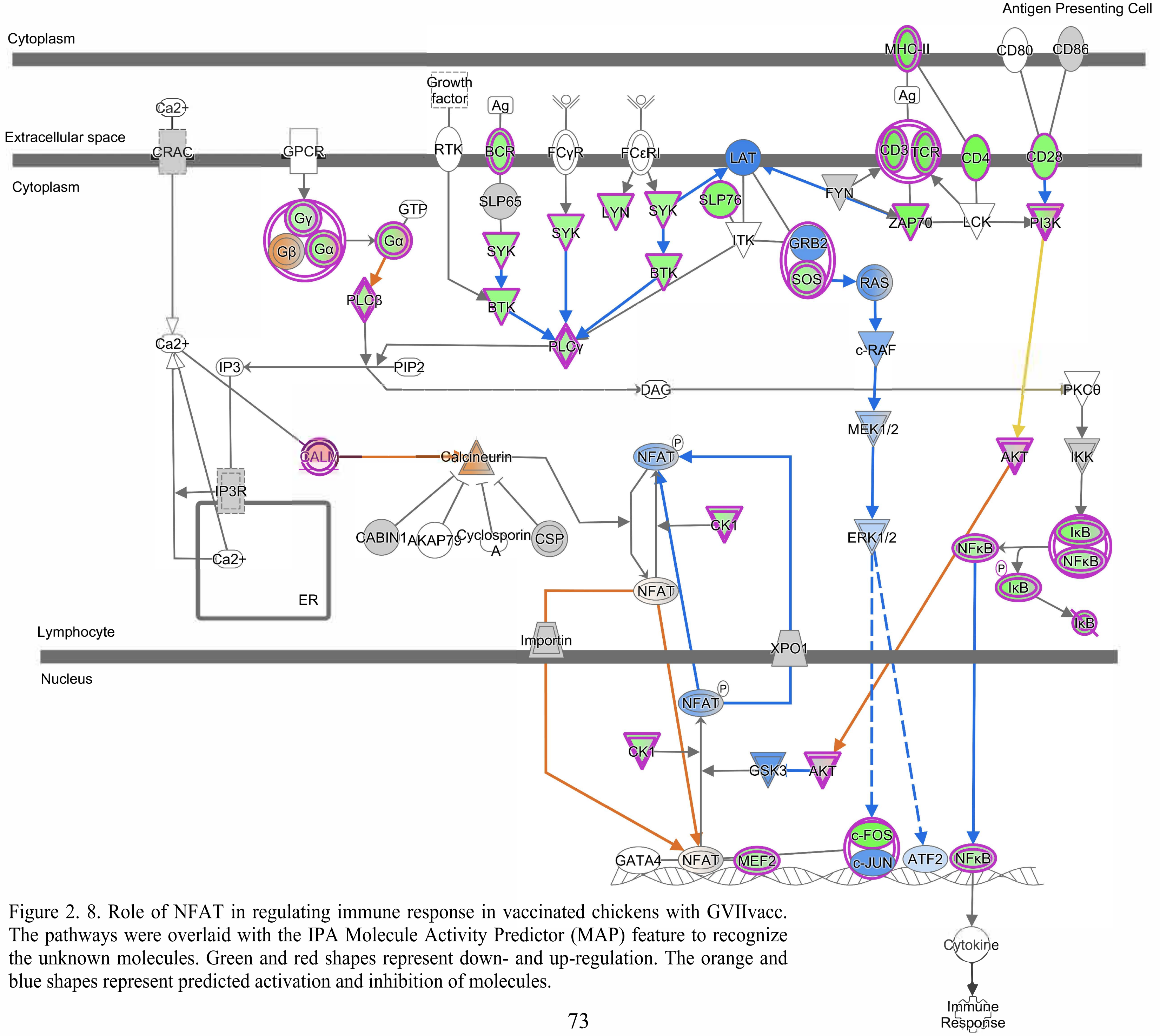
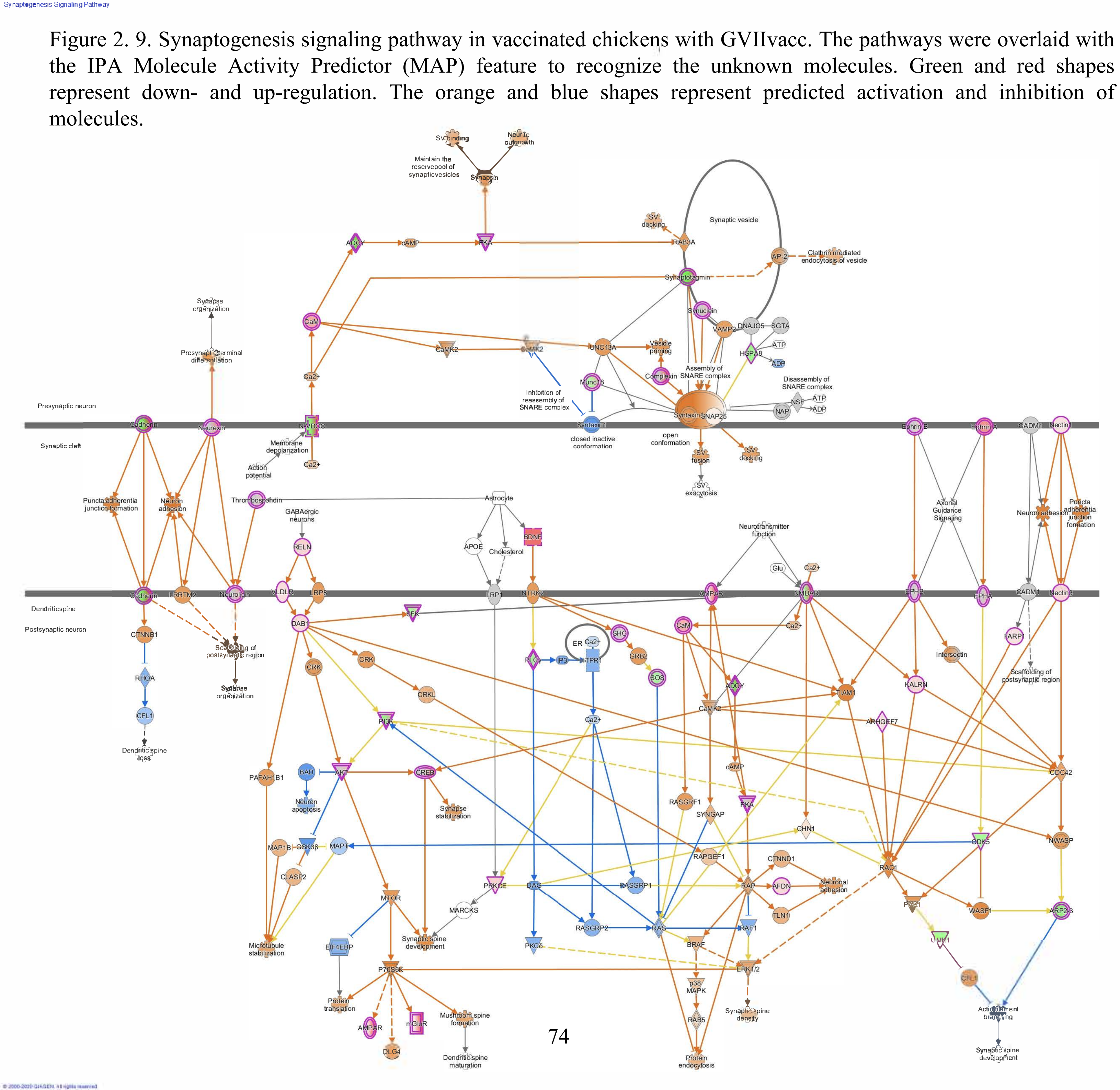


Figure 2. 8. Role of NFAT in regulating immune response in vaccinated chickens with GVIIvacc. The pathways were overlaid with the IPA Molecule Activity Predictor (MAP) feature to recognize the unknown molecules. Green and red shapes represent down- and up-regulation. The orange and blue shapes represent predicted activation and inhibition of molecules.

Figure 2. 9. Synaptogenesis signaling pathway in vaccinated chickens with GVIIvacc. The pathways were overlaid with the IPA Molecule Activity Predictor (MAP) feature to recognize the unknown molecules. Green and red shapes represent down- and up-regulation. The orange and blue shapes represent predicted activation and inhibition of molecules.



2. 3. Discussion

This study analyzed transcriptomes from the spleens of chickens vaccinated with either GII or GVII to determine the main differences in chicken responses at the genes and pathway level and is, to our knowledge, the first such analysis in vaccinated chickens. There were significant qualitative differences in molecular signatures between the two vaccinated groups when compared to controls. GVIIvacc modulated the canonical pathways that correlate with immune system regulation, including neuroinflammation signaling pathways and the other responses such as the synaptogenesis pathway. Specifically, GVIIvacc inhibited the neuroinflammation signaling pathway, especially NF-ƙB as a regulator of the inflammation process but stimulated the synaptogenesis pathway to activate more intracellular signaling cells, including LTP, LTD, and cAMP, in activating CREB as a transcription factor. Thus, although this analysis was carried out in the spleen, it is reasonable to conclude that GVIIvacc also affects the nervous system. This finding supports a previous study that this strain likely behaves as a neurotrophic virus [27]. Moreover, the connection between the peripheral nerve in the spleen with the central nucleus of the amygdala (CeA) and the paraventricular nucleus (PVN) in the brain [28] might be the bridge to trigger the signaling pathways in the brain. In contrast, GII did not invoke those pathways correlated with the nervous system function but regulated pathways that could prevent apoptosis, especially in B cells. The mechanism of apoptosis inhibition involved the down-regulation of PERK, part of EIF2α, to inhibit apoptosis-inducing ATF4/CHOP. In addition, GIIvacc inhibited cytokine signaling and leukocytes extravasation pathway as part of immune regulation in chicken.

NF-ƙB was suppressed by GVIIvacc. NF-ƙB is a central component of the neuroinflammation pathway that would protect the neuron system by suppressing pro-inflammation and activating neurotrophin [29-31]. In general, the neuroinflammation pathway plays a crucial role in maintaining homeostasis in the nervous system by strengthening synapses, sculpting circuits, and determining nervous system activity [32,33]. This dataset showed that the cells involved in neuroinflammation are microglia, astrocytes, and neurons, which regulate nervous system activity, including the immune response (Figure 2. 6). Microglia are central nervous system (CNS) specific macrophages comprising 10% of the brain population [34] and play a significant role as the brain parenchyma housekeeper [35]. Usually, NF-ꞵB stimulates the expression of genes encoding pro-inflammatory proteins, i.e., IL6, TNFα, IL-1β, IL18, and IL12. In pathological conditions, increased pro-inflammatory cytokines such as IL-1β, IL6, and TNFα cause brain damage due to ischemia associated with BBB disruption [36-38]. However, this dataset showed that down-regulated NF-ꞵB suppresses IL-1β, IL6, and TNFα so that BBB disruption, which results in neuronal damage, was inhibited (Figure 2. 6). Pro-inflammatory factors IL-12 and IL-18 were inhibited in chicken vaccinated with genotype VII leading to inhibit T cell recruitment into the nervous system. Also, IFN γ in astrocytes was down-regulated (Figure 2. 6), reducing T cell recruitment, microglia activation, CD4 +, and CD8 + cell activation. In physiological conditions, leukocytes such as T cells, macrophages, and dendritic cells are restricted in the brain parenchyma and only circulate in subarachnoid space, vascular, and cerebrospinal fluid [39]. Intracellular adhesion, which also plays a role in T cell recruitment in the neuronal system [40], is also inhibited due to down-regulated NF-ꞵB in astrocytes (Figure 2. 6). On the other hand, the down-regulated NF-ꞵB stimulates downstream neurotrophin activation, such as NTF3, indirectly activating neuron survival proteins shown with orange shape (Figure 2. 6). NTF3 is a neuronal protector, preventing apoptosis in cortical neurons [31]. Moreover, pathway components CASP8 and CASP3 were down-regulated to deactivate proteins that cause neuron damage and apoptosis. Some studies revealed that down-regulated NF-ꞵB has a role as a neuron protector by reducing inflammation and pain due to several nervous diseases, such as autoimmune encephalitis [29,30]. Thus, we speculated that the down-regulated NF-ᴋB has a neuroprotective role by preventing BBB disruption from leukocytes and cytokines and increasing the production of proteins useful for neuron survival.

Another significant pathway influenced by GVIIvacc was the synaptogenesis signaling pathway. The synaptogenesis pathway controls how neurons communicate with each other, including astrocytes and microglia, using neurotransmitters [32]. In this data set, the neurotransmitter was glutamate, an excitatory neurotransmitter in the nervous system [41]. Glutamate is released in the synaptic cleft and interacts with its receptors in post-synaptic neurons. The glutamate receptor has two classes, known as metabotropic glutamate receptor (mGluR) and ionotropic glutamate receptor (iGluR) [42]. iGluR class receptors such as N-Methyl-D-Aspartate Receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazole Propionic Acid Receptor (AMPAR) at postsynaptic neurons were affected in chickens vaccinated with the GVIIvacc (Figure 2. 9). NMDAR was deactivated while AMPAR was activated, possibly to maintain neuronal homeostasis, and because when both receptors are activated, neurotoxicity results [43]. In addition, activation of the NMDAR has a role in excitotoxic neuronal death by increasing the Ca^{2+} influx in cells [44]. Also, excessive glutamate causes neurotoxicity [34]. In this dataset (not depict), GRM7, a metabotropic glutamate receptor, was up-regulated and would capture the excessive amounts of glutamate at the cleft synapse and brought into pre-synaptic neurons by the solute carrier family, namely SLC1A and SLC17A. In GVIIvacc, when neurotransmitters interacted with their targets on post-synaptic neurons, these interactions activated intracellular signaling pathways, including LTD, LTP, calcium signaling, and cAMP signaling pathways. Altogether these pathways activate the transcription factor, CREB. LTP is expressed after phosphorylation of AMPAR by caMK II [45,46]. Initially, NMDAR in post-synaptic neurons removes Mg^{2+} ions to open the channel for Ca^{2+} so that calcium from the extracellular enters and activates intracellular Ca^{2+}-dependent signaling [47]. Ca^{2+} contributes to post-synaptic kinase/phosphatase signaling balance that can regulate LTP and LTD [48]. CREB is a transcription factor activated by binding between Ca^{2+} and caMK II through PKA/cAMP signaling [49,50]. CREB affects long-term synaptic efficacy [45] and is predicted to play a role in cell neuron survival [51]. However, for LTP, TMM is expressed after AMPAR dephosphorylation by protein phosphatase-1 (PP1) [52]. Cooperation between LTP and TMM with their opposite functions is crucial in synaptic plasticity [53]. We contemplated that regulation of neuron interactions and the balance of glutamate as a neurotransmitter in stimulating other intracellular signaling pathways such as CREB, LTP, and LTD pathways are the properties of the genotype VII vaccine in maintaining the nervous system homeostasis.

We predicted that the down-regulated neuroinflammation signaling pathway and the up-regulated synaptogenesis signaling pathway are hallmarks of the GVIIvacc. With down-regulated neuroinflammation, it activates proteins that play a role in neuronal survival, and by activating synaptogenesis, the balance of homeostasis in the nervous system can be maintained. Therefore, the use of the vaccine from genotype VII could be a consideration in preventing NDV outbreaks in countries where genotype VII is endemic to NDV. Furthermore, the evidence at the molecular level in this study supports evidence from previous studies in serology and clinical sign level [54-56] that the genotype VII vaccine as a homologous vaccine effectively prevents an NDV outbreak.

In contrast, GIIvacc affected the immune response, including cytokine signaling pathway and other responses such as cellular stress and injury genes pathway in chicken that not involved with the nervous system. The UPR pathway, which correlates to apoptosis, was down-regulated in GIIvacc DEG. This pathway regulates Endoplasmic Reticulum (ER) stress conducted by three transducers: PERK, IRE1, and ATF6, as a physiological or pathological response [57,58]. These three transducers participate in the apoptosis process. However, PERK as the central regulator of ER stress will determine the fate of cells due to their excessive stimulation [57]. In this GIIvacc DEG list, PERK, which is part of the EIF2α, was down-regulated, suppressing downstream ATF4/CHOP and preventing apoptosis, especially in B cells. Inhibition of apoptosis may be an advantage of the genotype II vaccine in preventing severe necrosis in lymphoid tissue and gastrointestinal, unlike the action of NDV genotype VII in a previous study [17]. Moreover, proteins encoded by genes such as XBP-1, IL-6, and cdc42, which have roles in the development, differentiation, and survival of plasma cells, were down-regulated by GIIvacc. For UPR, XBP-1 splicing mediated by IRE1 can induce differentiation of B cells, resulting in immunoglobulin expression and inducing IL-6 synthesis and secretion for plasma cell survival [59]. In our study, however, IRE1 and XBP-1 were down-regulated. Besides Rac and Rho, cdc42 is a part of the GTPases of the Rho family that regulated actin cytoskeleton dynamic, including in leukocytes [60,61]. Cdc42 is a pivotal regulator for B cell differentiation into plasma cells and the production of humoral antibodies [62]. However, in GIIvacc DEG, cdc42 was down-regulated. Deficiency of cdc42 in B cells reduces mature B cell motility and their ability to interact with T cells and inhibits B cell differentiation into antibody-producing cells [63]. Thus, the downregulated UPR, cytoskeleton regulation, and GTPase signaling were molecular signatures of GIIvacc.

Molecular signatures of both vaccines were predicted by using IPA software. The database of knowledge in this software is derived from the accumulated knowledge of humans and mice. When these signatures are used in other species, caution should be used in interpreting the results. Further investigations are needed to validate the role of predicted genes and pathways. For example, GVIIvacc behaves differently in terms of the effect of the nervous system in chicken, including the synaptogenesis pathway in maintaining the homeostasis among neurons. However, how synaptogenesis is involved in nervous system protection if there is NDV infection needs further study. Another prediction that arose concerned XBP-1, IL-6, and cdc42 correlated with plasma cell differentiation and survival in producing antibodies, and in chickens vaccinated using the NDV genotype II vaccine were down-regulated. This prediction raised interesting concerns regarding antibody production.

In conclusion, based on predictions from transcriptomic data sets using IPA software, the key difference is that the GVIIvacc activated neuroprotective activity, whereas the GII vaccine did not. Therefore, the application of the NDV vaccine from LaSota should not be considered in areas where GVII is the dominant genotype that causes outbreaks in the field. By revealing how both NDV vaccines interacted with the host at the gene level, this work provides an opportunity to produce new vaccines that affect specific genes or pathways essential to the chicken immune system. In addition, several genes or molecules that affect chicken immunity can be used as vaccine biomarkers to determine a vaccine's efficacy.

2. 4. Methods

2. 4. 1. Viruses and Vaccines

Two viral stocks were used. LaSota-GII, a common strain used for vaccines in Indonesia, was provided by major vaccine producers worldwide. Chicken/Indonesia/VD/003WJ/11, hereafter referred to as GVII, which caused an outbreak in 2011 in Indonesia [64], was obtained from the Indonesian Research Centre for Veterinary Science repository (BBALITVET). Each was propagated using 9-day-old SPF embryonated chicken eggs [65]. Haemagglutination (HA) test and qPCR were used to confirm the presence of NDV in the allantoic fluid. Reed and Muench's method was used to calculate 50% embryo infection dose (EID_{50}) as the required virus titer for vaccine production [66,67]. Sterile Phosphate-Buffered Saline (PBS) pH 7.2 was used to adjust the titer to 10^7 EID_{50} in each dose of the vaccines [55]. To inactivate the diluted viruses, 0.12% formalin was utilized for 12 hours at 4°C [68,69]. Each vaccine dose contained 0.5 ml incomplete Freund's adjuvant and 0.5 ml inactivated virus.

2. 4. 2. Animal Experiments

This study was approved by the ethics committee of the Indonesian Research Centre for Veterinary Science, Indonesia. All experiments were performed at its Biosafety Level 3 (BSL-3) facility based on the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Thirty, three-week-old specific-pathogen-free (SPF) chickens were divided into three groups of 10 and placed in separate isolators. All chickens were raised with *ad libitum* access to feed and water. The first group was used as a control group with no vaccine given (n=10). The second group was vaccinated with the GII vaccine, hereafter referred to as GIIvacc (n=10). The last group was vaccinated using the GVIIvacc (n=10). Treatment groups were immunized with vaccines on day 14 and day 28. On day 42, all the groups were euthanized using CO_2. From each bird, 100 mg of spleen was taken and immediately stored in 1 ml RNAlater^{TM} in a 2-ml tube (ThermoFisher Scientific) at −80℃.

2. 4. 3. RNA Preparation and Sequencing

Total RNA was extracted from spleen samples using *mir*Vana™ miRNA Isolation Kit based on manufacturer’s instructions (ThermoFisher Scientific, Lithuania). The quantity of RNA was measured using a NanoDrop 1000 Spectrophotometer v 3.8 (ThermoFisher Scientific). Library preparation and sequencing were performed at the Australian Genome Research Facility (AGRF). RNA quality was measured using a LabChip GX Touch nucleic acid analyzer (PerkinElmer, U.S.A.). The range of RNA quality was 5.6-10.0. Next, complementary DNA (cDNA) libraries were prepared using a KAPA-stranded RNA-seq kit (Roche, U.S.A.). Finally, the libraries were sequenced using an Illumina NovaSeq with a NovaSeq 6000 S4 Reagent Kit.

2. 4. 4. Data Analysis

The quality of raw data was assessed using FASTQC v 0.11.4 [70] and trimmed with TrimGalore v 0.4.2 [71] to a minimum length of 100 bp and a minimum sequencing quality of Phred score 10. Sequencing adapters were removed with AdapterRemoval v 2.2.1 [72]. The reads were aligned to the chicken reference genome (GRCg6a) using Hisat2 v 2.2.1 [73], sorted, merged, and indexed using SAMtools v 1.8 [74]. FeatureCounts was used to summarize counts of reads mapped to genes using Ensembl Annotation v 97 [75]. Voom-limma was used to compare samples grouped by vaccination status [76,77]. Read counts were converted to counts per million (CPM), and only genes with more than 1 CPM in at least three samples were kept. Counts were normalized by log-transforming the CPM after correcting for differences in library size. Counts were further normalized using M values' trimmed mean (TMM) [78]. Samples and individual observational levels of each expressed gene were weighted using voom to adjust for heterogeneity in their expression level [77]. Differentially expressed genes (DEGs) between groups were identified using a False Discovery Rate (FDR) of less than 0.05. The DEGs from the three group comparisons were analyzed using Ingenuity Pathway Analysis (IPA) software from QIAGEN to find significantly differentially expressed genes and pathways that correlate with the chicken immune response.

2. 4. 5. Validation of Gene Expression Using quantitative PCR (qPCR)

Seven DEGs from either vaccine group with an absolute Log Fold Change (LFC) greater than 1.6 were chosen for validation by quantitative PCR (qPCR). Primer-BLAST was used to find specific primers for each gene. The primers were designed to span exon-exon junctions to avoid amplification of genomic DNA. The range of PCR products was ~70-250 base pairs. The optimal range of primer melting temperature was $58-64℃$. RNA was converted to cDNA using a SuperScript IV Reverse Transcriptase kit (ThermoFisher Scientific), following the protocol recommended by the manufacturer. Quantitative PCR was performed used a

QuantiNova SYBR® Green RT-PCR kit (Qiagen, Germany). The ΔΔCt method was used to quantify the expression using log fold change. GAPDH and ACTB were used as reference genes for normalization. The relationship between the expression of the genes from the transcriptome and qPCR was estimated using Pearson correlation.

2. 5. Data Availability

The RNA-Seq data is available in the NCBI SRA database under the BioProject PRJNA675698.

2. 6. Acknowledgments

This project was supported by the Australian Centre for International Agricultural Research Research (ACIAR) with grant number AH/2015/003. The authors are grateful to Dr. Indro Cahyono, Mrs. Risa Indriani, and Dr. Simson Tarigan in BALITVET for research materials and facilitating the use of BSL-3 labs. Thanks to PT Caprifarmindo Laboratories for providing the SPF chickens and eggs. Thank you to Dr. Corrie Brown and Dr. Patti Miller, University of Georgia, the USA, for giving scientific opinions about this paper. The University of Adelaide, South Australia, for letting us utilize the PC 2 laboratory in Roseworthy.

2. 7. Author Contributions

P.P. prepared samples, performed IPA analysed, interpretated results, summarized results, and manuscript writing. A.P. validation and manuscript editing. R.T. conducted bioinformatics analysis, validation, reviewed, and edited manuscript. W.L conducted bioinformatics analysis, reviewed, and edited manuscript, performed visualization. Y.R. conducted bioinformatics analysis. P.K., and N.D. collected and prepared samples. F.H. designed and directed the study, obtained financial support, validation, and editing. All the authors reviewed, approved, and contributed to the final version of the manuscript.

2. 8. Competing Interests

The authors declare no competing interests.

2. 9. References

1 Rushton, J. & Gilbert, W. The economics of animal health: direct and indirect costs of animal disease outbreaks. *Working Document* **84** (2016).

2 Rima, B. *et al.* ICTV Virus Taxonomy Profile: Paramyxoviridae. *Journal of General Virology*, jgv001328 (2019).

3 Miller, P. J. *et al.* Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Infection, genetics and evolution* **29**, 216-229 (2015).

4 Dimitrov, K. M. *et al.* Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, Genetics and Evolution* **74**, 103917 (2019).

5 Kim, S.-H. *et al.* (Am Soc Microbiol, 2012).

6 Pandarangga, P. *et al.* Full-Genome Sequences of Two Newcastle Disease Virus Strains Isolated in West Java, Indonesia. *Microbiology Resource Announcements* **9** (2020).

7 Lomniczi, B. *et al.* Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). *Archives of virology* **143**, 49-64 (1998).

8 Pandarangga, P. *et al.* Pathogenesis of new strains of Newcastle disease virus from Israel and Pakistan. *Veterinary pathology* **53**, 792-796 (2016).

9 Dimitrov, K. M., Afonso, C. L., Yu, Q. & Miller, P. J. Newcastle disease vaccines-A solved problem or a continuous challenge? *Vet Microbiol* **206**, 126-136, doi:10.1016/j.vetmic.2016.12.019 (2017).

10 Diel, D. G. *et al.* Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection, genetics and evolution* **12**, 1770-1779 (2012).

11 Xiao, S. *et al.* (Am Soc Microbiol, 2012).

12 Hu, S. *et al.* A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. *Vaccine* **27**, 904-910 (2009).

13 Hu, Z. *et al.* Generation of a genotype VII Newcastle disease virus vaccine candidate with high yield in embryonated chicken eggs. *Avian diseases* **55**, 391-397 (2011).

14 Sultan, H. A. *et al.* Protective efficacy of the Newcastle disease virus genotype VII–matched vaccine in commercial layers. *Poultry science* **99**, 1275-1286 (2020).

15 Roohani, K. *et al.* Characterisation of genotype VII Newcastle disease virus (NDV) isolated from NDV vaccinated chickens, and the efficacy of LaSota and recombinant genotype VII vaccines against challenge with velogenic NDV. *Journal of veterinary science* **16**, 447 (2015).

16 Cornax, I., Miller, P. J. & Afonso, C. L. Characterization of live LaSota vaccine strain–induced protection in chickens upon early challenge with a virulent Newcastle disease virus of heterologous genotype. *Avian diseases* **56**, 464-470 (2012).

17 Susta, L. *et al.* Pathologic characterization of genotypes XIV and XVII Newcastle disease viruses and efficacy of classical vaccination on specific pathogen-free birds. *Veterinary pathology* **52**, 120-131 (2015).

18 Arachchige, S. N. K. *et al.* Transcriptomic analysis of long-term protective immunity induced by vaccination with Mycoplasma gallisepticum strain ts-304. *Frontiers in immunology* **11** (2020).

19 Lee, C.-C., Kim, B.-S., Wu, C. C. & Lin, T. L. Bursal transcriptome of chickens protected by DNA vaccination versus those challenged with infectious bursal disease virus. *Archives of virology* **160**, 69-80 (2015).

20 Sachan, S. *et al.* Adjuvant potential of resiquimod with inactivated Newcastle disease vaccine and its mechanism of action in chicken. *Vaccine* **33**, 4526-4532 (2015).

21 Pezeshki, A., Ovsyannikova, I. G., McKinney, B. A., Poland, G. A. & Kennedy, R. B. The role of systems biology approaches in determining molecular signatures for the development of more effective vaccines. *Expert review of vaccines* **18**, 253-267 (2019).

22 Li, S. *et al.* Molecular signatures of antibody responses derived from a systems biology study of five human vaccines. *Nature immunology* **15**, 195-204 (2014).

23 Pulendran, B., Li, S. & Nakaya, H. I. Systems vaccinology. *Immunity* **33**, 516-529 (2010).

24 Nakaya, H. I. *et al.* Systems biology of vaccination for seasonal influenza in humans. *Nature immunology* **12**, 786 (2011).

25 Querec, T. *et al.* Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *Journal of Experimental Medicine* **203**, 413-424 (2006).

26 Conesa, A. *et al.* A survey of best practices for RNA-seq data analysis. *Genome biology* **17**, 13 (2016).

27 Munir, M. *et al.* (Am Soc Microbiol, 2012).

28 Zhang, X. *et al.* Brain control of humoral immune responses amenable to behavioural modulation. *Nature* **581**, 204-208 (2020).

29 Brambilla, R. *et al.* Transgenic inhibition of astroglial NF-κB protects from optic nerve damage and retinal ganglion cell loss in experimental optic neuritis. *Journal of neuroinflammation* **9**, 1-12 (2012).

30 Brambilla, R. *et al.* Transgenic inhibition of astroglial NF-κB improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation. *The Journal of Immunology* **182**, 2628-2640 (2009).

31 Liot, G. *et al.* Neurotrophin-3-induced PI-3 kinase/Akt signaling rescues cortical neurons from apoptosis. *Experimental neurology* **187**, 38-46 (2004).

32 Nisticò, R. *et al.* Synaptoimmunology-roles in health and disease. *Molecular brain* **10**, 1-12 (2017).

33 Marin, I. & Kipnis, J. Learning and memory… and the immune system. *Learning & memory* **20**, 601-606 (2013).

34 Colonna, M. & Butovsky, O. Microglia function in the central nervous system during health and neurodegeneration. *Annual review of immunology* **35**, 441-468 (2017).

35 Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314-1318 (2005).

36 Clark, W. M., Rinker, L. G., Lessov, N. S., Hazel, K. & Eckenstein, F. Time course of IL-6 expression in experimental CNS ischemia. *Neurological research* **21**, 287-292 (1999).

37 Liu, T. *et al.* Tumor necrosis factor-alpha expression in ischemic neurons. *Stroke* **25**, 1481-1488 (1994).

38 Yamasaki, Y. *et al.* Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. *Stroke* **26**, 676-681 (1995).

39 Engelhardt, B., Vajkoczy, P. & Weller, R. O. The movers and shapers in immune privilege of the CNS. *Nature immunology* **18**, 123 (2017).

40 Greenwood, J. *et al.* leucocyte–endothelial cell crosstalk at the blood–brain barrier: a prerequisite for successful immune cell entry to the brain. *Neuropathology and applied neurobiology* **37**, 24-39 (2011).

41 Zhou, Y. & Danbolt, N. C. Glutamate as a neurotransmitter in the healthy brain. *Journal of neural transmission* **121**, 799-817 (2014).

42 Traynelis, S. F. *et al.* Glutamate receptor ion channels: structure, regulation, and function. *Pharmacological reviews* **62**, 405-496 (2010).

43 Choi, D. W. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**, 623-634 (1988).

44 Choi, D. W. Glutamate receptors and the induction of excitotoxic neuronal death. *Progress in brain research* **100**, 47-51 (1994).

45 Poncer, J. C., Esteban, J. A. & Malinow, R. Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by α-Ca2+/calmodulin-dependent protein kinase II. *Journal of Neuroscience* **22**, 4406-4411 (2002).

46 Park, M. AMPA receptor trafficking for postsynaptic potentiation. *Frontiers in cellular neuroscience* **12**, 361 (2018).

47 Soderling, T. R. & Derkach, V. A. Postsynaptic protein phosphorylation and LTP. *Trends in neurosciences* **23**, 75-80 (2000).

48 Lee, H.-K. Synaptic plasticity and phosphorylation. *Pharmacology & therapeutics* **112**, 810-832 (2006).

49 Silva, A. J., Kogan, J. H., Frankland, P. W. & Kida, S. CREB and memory. *Annual review of neuroscience* **21**, 127-148 (1998).

50 Deisseroth, K., Bito, H. & Tsien, R. W. Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron* **16**, 89-101 (1996).

51 Walton, M. R. & Dragunow, M. Is CREB a key to neuronal survival? *Trends in neurosciences* **23**, 48-53 (2000).

52 Bliss, T. V. & Cooke, S. F. Long-term potentiation and long-term depression: a clinical perspective. *Clinics* **66**, 3-17 (2011).

53 Brini, M., Calì, T., Ottolini, D. & Carafoli, E. Neuronal calcium signaling: function and dysfunction. *Cellular and molecular life sciences* **71**, 2787-2814 (2014).

54 Miller, P. J., King, D. J., Afonso, C. L. & Suarez, D. L. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. *Vaccine* **25**, 7238-7246 (2007).

55 Miller, P. J. *et al.* Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. *Developmental & Comparative Immunology* **41**, 505-513, doi:https://doi.org/10.1016/j.dci.2013.06.007 (2013).

56 Yang, H.-m., Zhao, J., Xue, J., Yang, Y.-l. & Zhang, G.-z. Antigenic variation of LaSota and genotype VII Newcastle disease virus (NDV) and their efficacy against challenge with velogenic NDV. *Vaccine* **35**, 27-32 (2017).

57 Liu, Z., Lv, Y., Zhao, N., Guan, G. & Wang, J. Protein kinase R-like ER kinase and its role in endoplasmic reticulum stress-decided cell fate. *Cell death & disease* **6**, e1822-e1822 (2015).

58 Janssens, S., Pulendran, B. & Lambrecht, B. N. Emerging functions of the unfolded protein response in immunity. *Nature immunology* **15**, 910 (2014).

59 Iwakoshi, N. N. *et al.* Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nature immunology* **4**, 321-329 (2003).

60 Etienne-Manneville, S. & Hall, A. Rho GTPases in cell biology. *Nature* **420**, 629-635 (2002).

61 Bokoch, G. M. Regulation of innate immunity by Rho GTPases. *Trends in cell biology* **15**, 163-171 (2005).

62 Burbage, M. *et al.* Cdc42 is a key regulator of B cell differentiation and is required for antiviral humoral immunity. *Journal of Experimental Medicine* **212**, 53-72 (2015).

63 Kapczynski, D. R., Afonso, C. L. & Miller, P. J. Immune responses of poultry to Newcastle disease virus. *Dev Comp Immunol* **41**, 447-453, doi:10.1016/j.dci.2013.04.012 (2013).

64 Farhid Hemmatzadeh, M. M. A., Esmaeil Ebrahimie, Simson Tarigan, Mohammad Indro Cahyono. Molecular characterisation of newly emerged Newcastle disease viruses in Indonesia (Australian Centre for International Agricultural Research (ACIAR), Canbera, Australia, 2016).

65 in *World Animal Health - 2012. Volume 1* (OIE (World Organisation for Animal Health), Paris; France, 2013).

66 Ramakrishnan, M. A. Determination of 50% endpoint titer using a simple formula. *World journal of virology* **5**, 85-86, doi:10.5501/wjv.v5.i2.85 (2016).

67 (FAO), F. a. A. O. *A Basic Laboratory Manual for the Small-Scale Production and Testing of I-2 Newcastle Disease Vaccine*, <http://www.fao.org/docrep/005/ac802e/ac802e00.htm#Contents> (2002).

68 Mahboob, T., M, A., H, A. & Siddique, M. *Preparation and Evaluation of Newcastle Disease Oil Emulsion Vaccine at Hydrophile Lipophile Balance 7.0 Using Mukteswar Strain*. (1999).

69 Reddy GS, S. V. Comparison of two experimental binary ethylenimine (BEI) inactivated Newcastle disease oil-emulsion vaccines. *Acta virologica* **35**, 3 (1991).

70 Andrews, S. (Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom, 2010).

71 Krueger, F. & Galore, T. (2015).

72 Schubert, M., Lindgreen, S. & Orlando, L. AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC research notes* **9**, 88 (2016).

73 Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature biotechnology* **37**, 907-915 (2019).

74 Li, H. *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).

75 Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923-930 (2014).

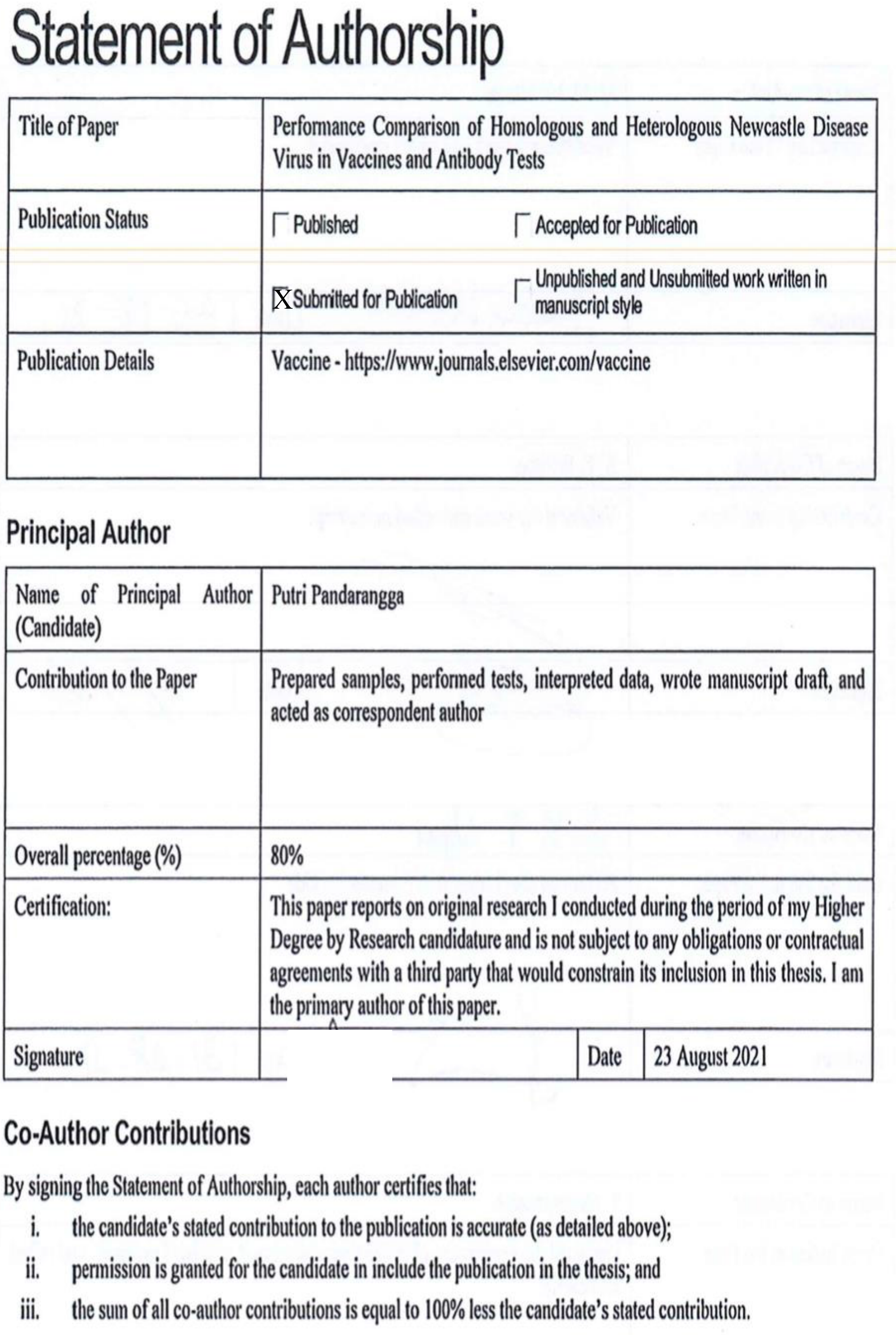
76 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research* **43**, e47-e47 (2015).

77 Liu, R. *et al.* Why weight? Modelling sample and observational level variability improves power in RNA-seq analyses. *Nucleic acids research* **43**, e97-e97 (2015).

78 Robinson, M. D. & Oshlack, A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome biology* **11**, 1-9 (2010).

CHAPTER 3

COMPARISON OF THE EFFICACY OF HOMOLOGOUS AND HETEROLOGOUS NEWCASTLE DISEASE VIRUS AS VACCINES DETECTED USING SEROLOGICAL RESPONSE



Statement of Authorship

Title of Paper	Performance Comparison of Homologous and Heterologous Newcastle Disease Virus in Vaccines and Antibody Tests
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Vaccine - https://www.journals.elsevier.com/vaccine

Principal Author

Name of Principal Author (Candidate)	Putri Pandarangga		
Contribution to the Paper	Prepared samples, performed tests, interpreted data, wrote manuscript draft, and acted as correspondent author		
Overall percentage (%)	80%		
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.		
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ii. permission is granted for the candidate in include the publication in the thesis; and
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Performance Comparison of Homologous and Heterologous Newcastle Disease Virus in Vaccines and Antibody Tests

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This manuscript is under review in the **Vaccine** Journal

Chapter 3: Comparison of the Efficacy of Homologous and Heterologous Newcastle Disease Virus as Vaccines Detected Using Serological Response

Abstract

Antigenic differences between commercial Newcastle Disease Virus (NDV) vaccine and circulating field virus are believed to contribute to reduced vaccine efficacy that enables breakthrough infections. In this study, fifty-layer chickens were divided into five groups: three groups of chickens vaccinated using killed LaSota (Genotype II or GII), Mega, or VD (Genotype VII or GVII) viral strains, and two control groups as the negative and positive control. On day 28, Hemagglutination Inhibition (HI) testing of sera from vaccinated chickens was performed with whole virus antigens of the Research Institute for Veterinary Science (RIVS), LaSota, Mega, and VD strains. Sera were also tested with alternative antigens, the cleavage site F protein peptide from GII and GVII NDV viral strains. Vaccinated birds and unvaccinated positive control birds underwent infectious challenges using VD and Mega strains. HI testing showed that antibody titers were higher when tested using homologous antigen in comparison with heterologous antigen. ELISA performed with cleavage site F protein peptides did not perform as well as the established HI test using homologous viral strains. On day 42, all groups except negative control group were challenged with GVII strain. The qPCR results showed that viral shedding was reduced by vaccination that was homologous to the infectious challenge in comparison with vaccination using the LaSota strain virus. Moreover, HI antibody titers induced by vaccines were predictive of the level of viral shedding two days after challenge infection. Thus, the vaccine, tested antigen, and challenge virus to test the effectiveness of the vaccine should be homologous with the dominant NDV circulating in the field to obtain the relevant antibody titer against NDV.

Keywords: Newcastle Disease Virus, Homologous Antigen, Fusion Protein Peptide, Homologous Vaccine, Protective Titer Antibody.

3. 1. Introduction

Newcastle Disease Virus (NDV) causes significant economic loss in the poultry industry worldwide. NDV is *Avian Orthoavulavirus* 1, which belongs to the genus *Orthoavulavirus* in the family *Paramyxoviridae* [1]. NDV is a single-stranded RNA virus with six genes: nucleocapsid protein (NP), polymerase-associated (P), matrix (M), fusion (F), haemagglutination-neuraminidase (HN), and large (L) that encode seven proteins. Six of these proteins have the same name as the genes, with the seventh being a V protein [2-4]. Based on amino acid characteristics of the F protein, NDVs are divided into class I and class II [5]. Class I has one genotype that contains non-virulent strains, except for the NDV strain that caused outbreaks in Ireland in 1990 [6]. Class II consists of 21 genotypes [7], of which genotypes V, VI, VII, and XIII are virulent [8-10]. In recent years, NDV genotype VII (GVII) has become the predominant strain in most countries in Asia, Europe, and Africa and has been responsible for numerous Newcastle Disease (ND) outbreaks [11-13]. However, the LaSota and B1 vaccines derived from the genotype II (GII) strain developed a few decades ago are the most widely used commercial vaccines worldwide [14-16].

After the NDV outbreaks in 2002 in the USA, which caused $200 million in losses, Kapczynski (2005) reported that the commercial vaccine in circulation could not prevent infection and viral shedding when chickens were challenged with virulent NDV [17]. Subsequently, NDV outbreaks were proposed to occur because of antigenic differences between the vaccine and the strain circulating in the field [18,19]. Moreover, developing a vaccine similar to a genotype that causes an outbreak in the field is recommended to prevent viral shedding [20]. To reduce viral shedding and prevent clinical signs, vaccines derived from GVII, the dominant cause of NDV outbreaks, have been developed [18,19,21].

However, several countries, including Indonesia, still rely predominantly on GII-derived LaSota commercial vaccines to combat outbreaks likely caused by GVII. According to the Indonesian veterinary drugs index (2017), the most commonly used NDV vaccine in Indonesia is the LaSota strain (representing 62% of all NDV vaccines), and only 0.4% of vaccines distributed in Indonesia contain the GVII virus, known as G7b [22]. Moreover, based on phylogenetic analysis, G7b actually contains a GI virus, not a GVII virus. The use of vaccines other than the LaSota vaccine is not popular in Indonesia, possibly because there is not enough locally relevant information to convince people that a different vaccine should be used. Thus, using vaccines homologous with NDV circulating in the field and testing the effectiveness of vaccines by challenge with prevalent NDV in the field are needed in Indonesia.

The haemagglutination inhibition (HI) test is globally the most commonly used serology test for assessing the antibody titers of chicken to NDV [23]. However, the reliability of HI tests has been questioned due to the variability of results when different antigens are used [24]. De Wit et al. (2007) evaluated HI test results from 120 laboratories worldwide [25] and showed that, even for tests undertaken using a protocol from World Organization for Animal Health, known as OIE, there were variations in the results due to different hemagglutinin (HA) antigens [25]. Indonesian veterinary laboratories have used the RIVS strain as a national standard for the HI test as antigen, a GI strain of NDV. Moreover, Miller et al. (2007) reported that sera from vaccinated chickens using GI, GII, GV, and GVI vaccines gave different antibody responses when tested with antigen from GI (Ulster strain) [21]. The HI test shows that employing HI antigens mismatched with vaccine and field strain genotypes is likely to provide unrealistic antibody titers and thereby misinform NDV surveillance and control programs. Therefore, we hypothesize that, in comparison with the RIVS virus, performing serology using circulating field strains of the virus as antigen will improve the discernment of protective antibody titers.

Not all veterinary laboratory facilities can safely produce wild-type virulent viruses for use as antigens in serology tests. As a result, the antigen(s) used in the serology test is limited to those arising from non-virulent strains. Thus, it would be valuable to develop antibody tests that use short peptides of the NDV F protein cleavage site rather than the whole virus. Furthermore, the cleavage site of F proteins has a significant role in initiating infection and determining the virulence of NDV [26]. For example, a 6-amino acid basic peptide virulence motif found at the F protein cleavage site of virulent NDV was reported to react with sera from chickens infected with virulent but not non-virulent NDV [27]. In addition, we want to compare antibody titers as measured using different viral strains and purified F antigens to compare their predictive value against viral challenge. Hence, this study investigates whether vaccines that use viral strains homologous with those that cause outbreaks in Indonesia can reduce viral shedding.

3. 2. Materials and Methods

3. 2. 1. Animal Experiments

All experiments were performed at the Indonesian Research Center for Veterinary Science, known as BBALITVET, where its Research Committee approved animal ethics. Fifty specific pathogen-free (SPF) layer chicks were reared in the biosafety level 3 (BSL3) animal house facility of BBALITVET, with no restrictions on water and food. These chickens were divided into five groups of 10 birds. When the birds were 15 days old, defined as day 0, the first, second, and third groups were vaccinated subcutaneously using one dose of the killed LaSota, Mega, and VD strains, respectively, and this was repeated 14 days later. The fourth group had no treatment, and the fifth group was unvaccinated and infected with virulent NDV. Each vaccine contained 10^7 Embryonic Infectious Dose$_{50}$ (EID$_{50}$). Sera from all chicken groups for ELISA and HI tests were obtained on day 28. Sera were heat-inactivated at 56℃ for 30 minutes and then stored at -80℃ until used. All vaccinated birds and a positive control group were challenged with virulent Mega and VD strains of NDV on day 42, while an unvaccinated negative control group was left unchallenged. At two days post-infection, cloacal swabs were obtained from all chickens for the quantitative testing of viral shedding before euthanized using CO_2.

3. 2. 2. Virus Isolation and Vaccines

BBALITVET provided four stocks of NDV isolates: RIVS (GI), chicken/LaSota/ACIAR (GII), chicken/Indonesia/Mega/001WJ (GVII), and chicken/VD/2011 (GVII) strains. Chicken/Indonesia/Mega/001WJ and chicken/VD/2011 were isolated from natural outbreaks of ND that occurred in LaSota-vaccinated birds in 2013 and 2011, respectively, and both strains have caused outbreaks in West Java [28,29]. The RIVS strain is used as the national standard antigen in HI tests in Indonesia, and the chicken/LaSota/ACIAR strain is used in commercial vaccines in Indonesia. The selected NDV isolates were plaque purified and propagated in SPF chicken eggs then stored in aliquots in -80℃ freezers. The chosen viruses were propagated in 10-day-old SPF embryonated chicken eggs [30]. HA test and qPCR were used to confirm the presence of NDV in the harvested allantois fluid. Allantois fluid was centrifuged at 8000g then filtered using 0.22 µM filters to purify the virus. The 50% embryo infection dose (EID$_{50}$) for each viral strain was determined using Reed and Muench's method [31,32]. To adjust the titer up to

10^7 EID_{50}, the purified virus was diluted in sterile phosphate-buffered saline (PBS) pH 7.2 [20]. Only LaSota, Mega, and VD NDV isolates were used to make vaccine seeds and then stored at -80℃ until used. A final concentration of 0.12% formalin was added to the virus and incubated at 4℃ for at least 12 hours to inactivate the diluted viruses [33,34]. Each vaccine contained 0.5 ml of the inactivated viruses mixed with 0.5 ml of incomplete Freund's adjuvant.

3. 2. 3. Hemagglutination-Inhibition (HI) test

The HI tests were performed based on the OIE protocol [35]. Briefly, 50 µl of a serial two-fold dilution of test sera in PBS was placed in each well of a U-bottom microplate. Next, a 50 µl NDV antigen containing four hemagglutination units (HAU) of the virus was added. RIVS, LaSota, VD, or Mega viruses were used as antigens. The plates were incubated for 45 minutes at room temperature. Next, an equal volume of 0.5% chicken red blood cells (RBCs) was added to each well then incubated for 45 minutes. The result was determined by finding the last dilution with complete inhibition of a hemagglutination reaction. The tests were run in duplicate.

3. 2. 4. Design of cleavage site F protein from genotype II (GIIF) and genotype VII (GVIIF) peptides as antigens

Two peptides corresponding to the F protein cleavage site of GII (GIIF) and GVII (GVIIF), respectively, were synthesized by ChinaPeptides Co. LTD (Shanghai-China) as antigens for use in an enzyme-linked immunoassay (ELISA). Each peptide was supplied as 1 mg of lyophilized peptide powder of more than 90% purity. The amino acid positions of the GIIF peptide were ^{106}SVTTSGGGRQGRLIGAIIGGV^{126} taken from LaSota F protein (AJ629062.1). In contrast, the amino acid positions for the GVIIF peptide were

^{105}SVSTSGGRRRKRFIGAVIGSV^{125} that were obtained from Mega F protein (MN688613). The GIIF and GVIIF peptides were dissolved in 1 ml of distilled water, then 50µl aliquots were stored at -80℃ until used.

3. 2. 5. ELISA Test for Cleavage Site F protein of GII and GVII Peptides

ELISA was performed as previously published by our group [36]. The heat-inactivated sera were centrifuged at 10,000 rpm for 10 minutes at 4℃ to remove any denatured particles, and the supernatant was used for further analysis (Eppendorf Centrifuge 5424R, Germany). The chicken sera were diluted at 1:80 in ELISA dilution buffer. A checkerboard titration using different positive serum dilutions and the GIIF and GVIIF peptides was used to optimize the ELISA. Based on the checkerboard titration, the peptides' optimal concentration was 50µg/100µL, and optimal serum dilution was 1/80.

Sera from unvaccinated chickens and those vaccinated with killed LaSota, Mega, or VD vaccines were tested with GIIF and GVIIF as antigens in ELISA. The peptides with stock concentration (25µg/µl) were diluted into carbonate buffer to get 50µg/100µL as the final concentration. The 100µl diluted antigens were dispensed into flat-bottom MICROLON® (Greiner bio-one, Germany) wells. Two uncoated wells were prepared as negative controls for each diluted serum to measure the possible noise from each serum sample. Each serum sample was tested in duplicate in the absence and presence of the antigens. The plate, covered with sealing film (Platemax, Axygen), was incubated at 4℃ overnight to coat the antigens. After incubation, wells were washed three times using ELISA washing buffer. 150 µl bovine serum albumin blocking buffer in 100 ml PBS was added to all wells then incubated for 2 hours at room temperature. After washing with ELISA washing buffer, 100 µl of 1/80 diluted chicken serum was added to each well. The plate was incubated for 1 hour at room temperature, then washed again with ELISA washing buffer. 50 µl of diluted horseradish peroxide-conjugated rabbit anti-chicken IgG (Sigma-Aldrich, USA) with ELISA buffer with concentration 1:1000 was added to wells. 100 µl 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich, USA) was added into all wells for 5-15 minutes. To stop the reaction, 50 µl 2M H_2SO_4 was added to each well. The result was read in a microplate spectrophotometer at a wavelength of 450 nm (xMark ™, BioRad).

3. 2. 6. Viral RNA extraction from Cloacal Swab and Primer Design

The RNA from the cloacal swab was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). Then, it was quantitated using the NanoDrop 1000 Spectrophotometer v 3.8 (ThermoFisher Scientific, USA). Next, viral RNA of NDV was converted to complementary DNA (cDNA) using the SuperScript^{TM} IV First-Strand Synthesis System (Invitrogen^{TM}, Lithuania) with poly(A) oligo(dT)$_{12\text{-}18}$ as a template/primer, based on the kit manual. The primers used to build PCR products with primer from the F gene (forward:5'-AAAGTGGTGACACAGGTCGG-3' and reverse: 5'-CCGATGTATTGCCGCTCAAG-3'). In addition, this gene was amplified using conventional PCR using the AllTaq ™ Master Mix Kit (Qiagen, Germany).

3. 2. 7. Quantification of viral shedding in qPCR by using Plasmid DNA as a Standard Curve

qPCR was used to calculate viral load in infected chickens with virulent NDV. Moreover, the standard curve used was plasmid DNA that cloned into *E. coli* TOPO 10 competent cells. All processes of this method follow the previous method [37]. The ct value was measured in triplicate to generate a standard curve. The ct values were plotted against the logarithm of serial dilution to cycle threshold [38]. By using the standard curve, viral shedding was determined. Ct values above 35 were considered negative [39].

3. 3. Statistical analysis

3. 3. 1. Hemagglutination Inhibition Analysis

All statistical analyses were performed in Stata version 15 (StataCorp, College Station, Texas). The reciprocal titer values from HI were log-transformed using the base-2 logarithm as follows:

$$Log_2\ titer(Y) = \log_2\left(\frac{reciprocal\ titer}{2}\right)$$

A base-2 logarithm was considered the most suitable transformation as serial two-fold dilutions were used to obtain the HI titrations. A linear mixed-effects model was used to examine vaccine effects (LaSota, Mega, and VD) and whole virus antigen (RIVS, LaSota, Mega, and VD) on $\log_2$ HI titer antibodies. Vaccine and antigen interaction was included by allowing the effect of the vaccine according to the antigen being tested. A random effect for chicken was included to account for the repeated measurements taken of individual birds. Mean titer values on the $\log_2$ scale for each combination of vaccine and antigen were obtained post-hoc. Planned contrasts assessing differences in antibodies were conducted between selected combinations of vaccine and antigen. Geometric Mean Titer (GMT) values were calculated from estimates on the log scale using the formula:

$$GMT = 2^{\hat{Y}} \times 2$$

Where $\hat{Y}$ is the estimate of interest on the log scale. Then, the estimated difference in $\log_2$ titer value of any two conditions will be approximately equal to the $\log_2$ Fold Change (FC) in reciprocal titer value:

$$\widehat{mean}(\log_2(condition\ A)) - \widehat{mean}(\log_2(condition\ B)) \approx log_2(\widehat{mean}\left(\frac{condition\ A}{condition\ B}\right) \approx \log_2(FC)$$

The estimate for $\log_2$ FC can then be back-transformed to represent Fold Change (FC) on the original scale for HI antibody:

$$Fold\ Change\ (FC) = 2^{\log_2(FC)}$$

The effect of vaccines (LaSota, Mega, and VD) on NDV infectious challenge (using Mega or VD) on qPCR ct values was examined using linear regression modeling with robust standard errors. In addition, an interaction between vaccine and challenge was included. Finally, mean qPCR ct values for each combination of vaccine and challenge were obtained post-hoc, and planned comparisons of interest were conducted.

3. 3. 2. ELISA Analysis

The steps used in analyzing ELISA results were almost identical to the statistical analysis for HI except for the specific antigens utilized. Instead of using a $\log_2$ titer, ELISA averaged two corrected ELISA Optical Density (OD) readings for each observation. In addition, a linear mixed-effects model was used to assess the effects of vaccination (LaSota, Mega, and VD) and F protein-peptide antigens (GIIF and GVIIF).

3. 3. 3. The relationship between vaccinal titers and viral shedding under challenge conditions

The relationship between vaccinal titers and viral shedding was assessed by comparing titer values ($\log_2$ HI values, log OD values) recorded for each vaccine-antigen combination with the qPCR ct value for the corresponding vaccine-challenge combination. The degree of linear association was quantified using Pearson's correlations and linear regression models. Moreover, the association was assessed graphically using scatterplots.

3. 4. Results

3. 4. 1. HI test results and comparison with ct values of viral shedding

As expected, sera from vaccinated chickens produced higher antibody responses against antigens homologous with the vaccine strain than against antigens from heterologous strains, as depicted in Figure 1. Specifically, the antibody response from Mega-vaccinated animals was higher for its homologous, Mega antigen ($\log_2$ HI titer = 2.13) than the LaSota GII antigen ($\log_2$=1.65). Similarly, for the VD-vaccinated birds, the antibody response was significantly higher for the VD antigen ($\log_2$ HI titer =1.99) compared to both the LaSota GII antigen ($\log_2$ HI titer =1.57) and the RIVS antigen ($\log_2$ HI titer =1.69). Finally, the antibody response from LaSota-vaccinated animals was significantly higher when exposed to its homologous, LaSota GII antigen ($\log_2$ HI titer =2.44), compared to exposure to either the Mega antigen ($\log_2$ HI titer = 1.78) or the VD antigen ($\log_2$ HI titer = 1.65). In sum, HI titers of the two GVII-vaccinated groups combined vs. the GII-vaccinated group were 1.23 (95% CI 1.15, 1.33) and 1.33 (95% CI 1.23, 1.42) times greater for the Mega GVII antigen and the VD GVII antigen, respectively.

Similarly, HI titers were on average 1.78 (95% CI 1.66, 1.92) times greater for GII-vaccinated animals relative to GVII-vaccinated animals for the LaSota antigen.

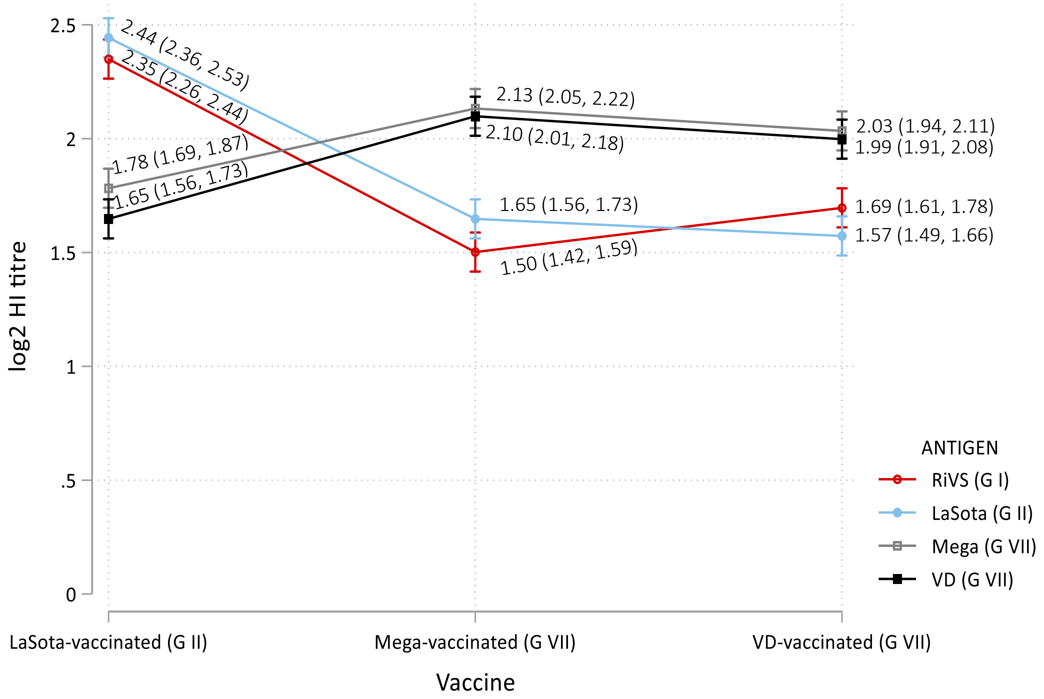


Figure 3. 1. Log_2 HI antibody titers from a linear mixed-effects model for each combination of vaccination and antigen, with 95% confidence intervals (CI).

Differences in the qPCR ct values between challenge groups are shown in Figure 2. The interaction between vaccine and challenge was significant (overall p=0.011). The mean level of viral shedding of LaSota vaccinated animals was similar for both the Mega challenge (mean ct value=24.90) and the VD challenge (mean ct value=23.71; p=0.600). However, for Mega-vaccinated animals, the estimated level of viral shedding was significantly lower in response to the Mega challenge than the VD challenge (31.0 vs. 26.0; p=0.001). For VD-vaccinated birds, the estimated mean ct value was 36.5 in response to the VD challenge, compared to 32.0 in response to the Mega challenge, which indicates a lower level of viral shedding. The difference in viral shedding between the two challenges among VD-vaccinated birds was not statistically significant (p=0.098).

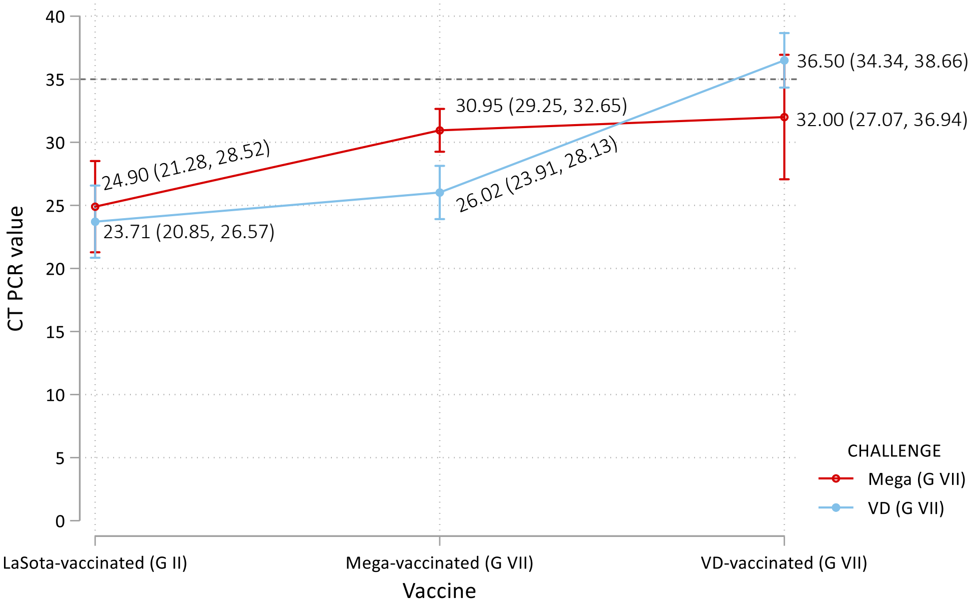


Figure 3. 2. The mean qPCR ct values for each challenge group for each vaccine from a linear regression model with 95% CI.

3. 4. 2. ELISA results and comparison with ct values of viral shedding

In general, log OD values of VD-vaccinated chickens are higher when tested with its homologous antigen, GVIIF protein (log OD value= 2.65), than heterologous antigen, GIIF protein (log OD value 1.98) depicted in Figure 4. Similarly, for Mega-vaccinated chicken, the log OD value was significantly higher towards GVIIF protein (log OD value= 2.90) than GIIF protein (log OD value= 2.34). However, the estimated log OD values among LaSota-vaccinated animals (log OD value= 3.37) were not significantly higher for the GIIF protein antigen (its homolog) compared to the GVIIF protein antigen ((log OD value= 2.91; p=0.055).

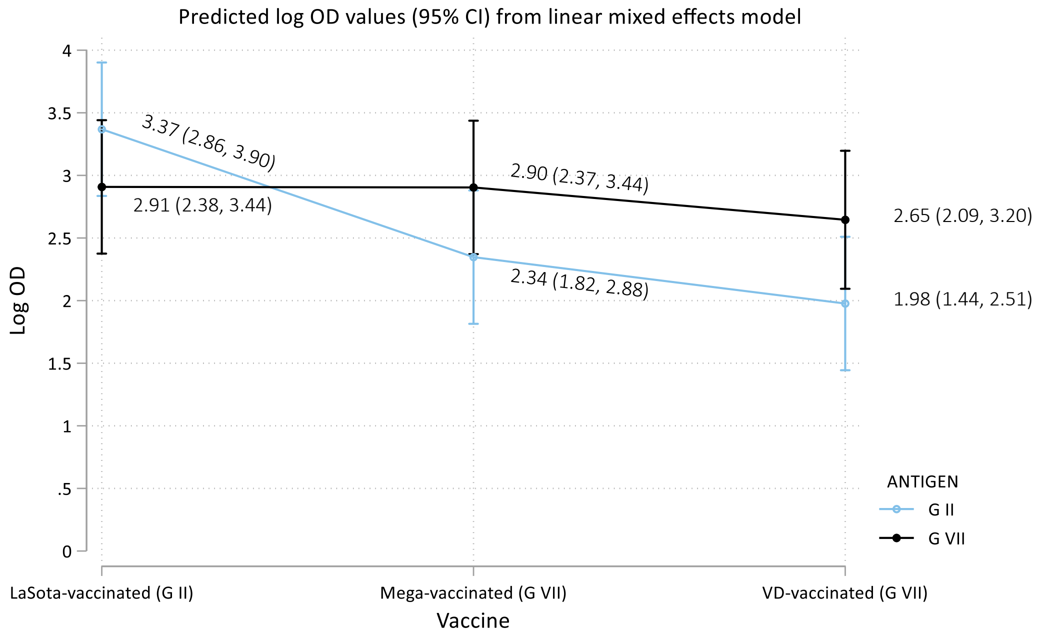


Figure 3. 3. Adjusted predictions for averaged ELISA log OD values of antibody titer from a linear mixed-effects model for each combination of vaccination and F protein antigen, with 95% CI.

3. 4. 3. Relationship between vaccinal titers and viral shedding under challenge conditions

Log$_2$ HI values were significantly associated with qPCR ct values (Pearson's r=0.5, p<.01), depicted in Figure 5. A one-unit increase in log$_2$ HI value was associated with an average increase of 13.9 units in the corresponding qPCR ct value (95% CI 5.48, 22.39; p=0.002). However, there was no significant association between log OD values (from ELISA) and the corresponding qPCR ct values detected (Pearson's r=0.3, p=0.17). Thus, the log OD value (from ELISA) was not a significant predictor of the corresponding qPCR ct value.

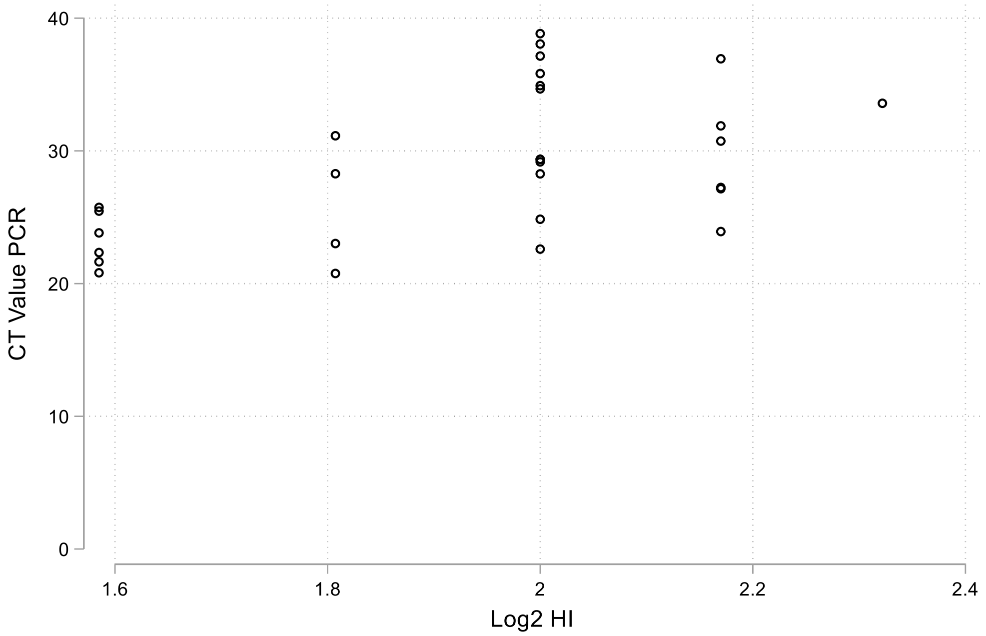


Figure 3. 4. Scatter plot between Log_2 HI titers and qPCR results with p<.01

3. 5. Discussion

The use of commercial NDV vaccines containing LaSota viral antigen is still common and represents 60% of the total NDV vaccines in circulation in Indonesia, while the dominant strain of NDV circulating in the field is derived from GVII such as Mega and VD strains [22]. The situation is similar in other Asian countries such as China [18]. Moreover, in Indonesia, the antigen for monitoring and evaluating the effectiveness of the NDV vaccines using the HI test is derived from genotype I or RIVS. But, when this genotype is used as an antigen to measure antibody responses in HI assays from different vaccines, it produces varying titers [21]. Thus, NDV outbreaks still occur frequently in Indonesia, even in chickens vaccinated with commercial vaccines, which maybe because of the phylogenic differences between the viruses used in vaccines and antibody tests and the prevalent type of NDV in the field [22,40]. Using heterologous antigens to evaluate the post-vaccination antibody titers makes a confusing situation for the animal health authorities. The proper measurement of protection antibody titers will be achieved when the circulating virus and the tested antigen match the HA test.

In this study, sera from three groups of chickens vaccinated with LaSota, Mega, and VD killed vaccines were tested using four different whole virus antigens, namely RIVS, LaSota, Mega, and VD in the HI test. It showed that the HI antibody titer from different vaccines varied significantly depending on the antigens being tested. Vaccines produced higher antibody responses against homologous viral antigens than against heterologous viral antigens. The three groups of vaccinated chickens were then challenged with Mega and VD strains. Quantitative PCR results demonstrated differences in protection against cloacal viral shedding between challenge groups depending on the viral strain used for vaccination. Viral shedding was decreased significantly in chickens administered vaccine that was homologous to the challenge strain. These results suggest that applying antigens and vaccines that are homologous with circulating strains in the field should be considered in Indonesia to measure relevant antibody titers and induce maximal reductions in viral shedding, respectively.

Our study also emphasizes that applying antigens similar to strains circulating in the field on HI assays is crucial in preventing NDV outbreaks in Indonesia. It has been demonstrated in a wide range of studies that the use of different antigens or different test conditions can affect the reliability of the HI test results [25]. The variability in results may be due to differences in HN proteins' immunogenicity between virulent and non-virulent NDV [41-43]. With immunogenicity potency, HN protein can induce protective HN-specific antibodies against NDV [44]. HN protein epitopes from non-virulent NDVs [45] are different from virulent NDVs [46-48]. In HN protein derived from GVII NDV, there are four antigenic domains of which the P2 domain, at position 53-192 amino acid (aa), has the strongest immunogenicity [41]. The antigenic domain on HN protein has been shown to stimulate HN-specific antibodies to protect chickens from death after a challenge with GVII NDV. The epitopes in HN proteins of virulent NDVs mutate rapidly, especially at the 347 position, providing different antigenic features for HN proteins [48]. The antigenic domain on non-virulent NDV, including LaSota, is always located at aa 513-569 [49]. It follows that only homologous antigens to NDV prevalent in the field can measure relevant antibody titers to NDV vaccines, as seen in the results of our study. The immunogenicity of the antigenic domain HN protein may trigger HN-specific antibodies that can be captured only by the epitope in the antigenic domain of homologous antigen. Therefore, using RIVS as a common antigen for HI assay in veterinary laboratories in Indonesia needs to be reconsidered because it is sensitive for detecting antibody titer for LaSota vaccine as a commercial vaccine but may not provide a sufficiently sensitive antibody titer for dominant circulating NDV, GVII, in the field.

This study showed that chickens vaccinated using homologous vaccines had reduced viral shedding after the challenge with virulent NDV strains circulating in Indonesia. These results are consistent with previous research suggesting that the homologous vaccines that are phylogenetically similar to prevalent NDV in the field can reduce viral shedding compared to commercial vaccines [18,19,21]. Also, our data showed that antibody responses from LaSota vaccinated animals were the highest of all vaccinated chicken groups when tested using its homologous antigen. However, this group of chickens shed virus significantly when challenged with GVII strains. This result does not agree with Miller [20] and Susta [50], who suggest that LaSota can still provide protection as long as the antibody titer exceeds the cut-off protection and can reduce viral shed after challenge with virulent NDV, respectively. So, it may be concluded that higher titers in serological tests do not necessarily represent better protection in vaccinated chickens when the vaccines and the antigens in serological tests are not well-matched to the circulating viruses in the field. The immunogenicity of the antigenic domain of the HN protein may be capable of stimulating HN-specific antibodies that can be captured only by the epitope in the antigenic part of the homologous antigen. Using the cross HI and virus neutralization tests, antibodies derived from vaccines with HN protein identical to the challenge strain can significantly reduce viral shedding compared to the LaSota vaccine [42]. The two strains, namely Mega and VD, are NDV virulent genotype VII which caused outbreaks in vaccinated broilers with a mean death time of 38 and 40 hours in Indonesia [22]. The results of our experiment show that HI titer levels are predictive of the level of viral shedding after infectious challenge ($p < 0.01$). Interestingly, the Mega vaccine comes from GVII, but it was not as good against VD challenges as the VD vaccine was against Mega challenges. Thus, based on the differences in the vaccines' ability to suppress viral shedding, we speculated that VD might be a better vaccine candidate than Mega.

Not all veterinary laboratory facilities can safely produce wild-type virulent viruses for use as antigens in serology tests. Therefore, this study developed a cleavage site of F protein-peptide and assessed its utility as an alternative test antigen in ELISA for measuring protective antibodies. Although the cleavage site of F protein has a role in determining virulence and inducing neutralizing antibodies [51], our data analysis showed that NDV peptide is not recommended as an alternative antigen in ELISA. Despite the fact that Mega and VD vaccinated animals' antibody response was higher for GVII F protein-peptide than GII F protein-peptide, there was no significant interaction between antibody titer to decrease viral shedding by chickens challenged with genotype VII strains. The antibody response was higher among Mega- and VD-vaccinated chickens than LaSota vaccinated chickens, possibly due to the different motifs of the cleavage site of F protein in virulent and non-virulent NDV. The cleavage-site motifs of virulent NDV are ^{112}RRQ/RKRF^{117}, while non-virulent NDV is ^{112}GKQGR↓L^{117} [52,53]. In addition, the high antibody response observed only in Mega and VD vaccinated animals may be due to the presence of epitope-specific antibodies which recognize the KRF motif in the F protein cleavage-site from virulent NDV [27]. However, captured antibodies from cleavage site F protein cannot be conclusively considered as protective antibodies. Pearson’s correlations showed little evidence for an association between F protein antibody titers and reductions in viral shedding. Hence, based on our analysis, the cleavage site F protein is not recommended as an alternative antigen in measuring antibody titers for monitoring and evaluating the effectiveness of the NDV vaccine in the field.

In conclusion, to combat NDV in Indonesia, it is recommended to use vaccines based on circulating field strains of the virus and to assess HI antibody titers of vaccinated chickens using homologous viral strains as test antigens. This could help address the issue of variability of HI tests in 120 laboratories [25]. However, further study is needed to examine the application of this method in the field and employing suitable antigens in serological tests may improve the assessment of vaccine efficacy and NDV surveillance. In addition, designing of HN peptide in ELISA is needed to investigate as a new antigen.

3. 6. Acknowledgments

We are grateful for funding from the Australian Centre International Agricultural Research (ACIAR) with project number AH/2015/003. BBALITVET's team (Dr. Niluh Dharmayanti, Mrs. Risa Indriani, and Dr. Simson Tarigan) for providing Animal House in Bogor. The University of Adelaide for providing significant support to publish this paper. Kandarp Patel for initiating the statistical analyses. Jana Bednarz for performing the statistical analyses. Dr. Indro Cahyono for the excellent laboratory assistance in Indonesia. Dr. Corrie Brown for proofreading this manuscript.

3. 7. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

3. 8. Authors' Contributions

PP conducted laboratory analyses, data interpretation, drafted or revised the article draft. AP, MM, and FH revised the draft of the manuscript and supervised the laboratory work. FH planned and designed the study. FH and MM performed *in vivo* procedures. YN performed the checkerboard for ELISA. All authors read and approved the final version of the article.

3. 9. References

1 Rima, B. *et al.* ICTV Virus Taxonomy Profile: Paramyxoviridae. *Journal of General Virology*, jgv001328 (2019).

2 Lamb, R. A. & Kolakofsky, D. in *Fundamental virology* (eds D. M. Knipe & P. M. Howley) (Lippincott Williams & Wilkins, Philadelphia; USA, 2001).

3 Kattenbelt, J. A., Stevens, M. P. & Gould, A. R. Sequence variation in the Newcastle disease virus genome. *Virus research* **116**, 168-184 (2006).

4 Steward, M., Vipond, I. B., Millar, N. S. & Emmerson, P. T. RNA editing in Newcastle disease virus. *Journal of General Virology* **74**, 2539-2547 (1993).

5 Liu, H. *et al.* Multiplex RT-PCR for rapid detection and differentiation of class I and class II Newcastle disease viruses. *Journal of virological methods* **171**, 149-155 (2011).

6 Alexander, D. J. *et al.* Characterisation of an antigenically unusual virus responsible for two outbreaks of Newcastle disease in the Republic of Ireland in 1990. *The Veterinary record* **130**, 65-68 (1992).

7 Dimitrov, K. M. *et al.* Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, Genetics and Evolution*, 103917 (2019).

8 Pandarangga, P. *et al.* Full-Genome Sequences of Two Newcastle Disease Virus Strains Isolated in West Java, Indonesia. *Microbiology Resource Announcements* **9** (2020).

9 Pandarangga, P. *et al.* Pathogenesis of new strains of Newcastle disease virus from Israel and Pakistan. *Veterinary pathology* **53**, 792-796 (2016).

10 Kim, S.-H. *et al.* Evaluation of fusion protein cleavage site sequences of Newcastle disease virus in genotype matched vaccines. *PloS one* **12** (2017).

11 Abolnik, C. *et al.* Tracing the origins of genotype VII h Newcastle disease in Southern Africa. *Transboundary and emerging diseases* **65**, e393-e403 (2018).

12 Rui, Z. *et al.* Phylogenetic characterization of Newcastle disease virus isolated in the mainland of China during 2001–2009. *Veterinary microbiology* **141**, 246-257 (2010).

13 Fuller, C. *et al.* An epizootiological report of the re-emergence and spread of a lineage of virulent Newcastle disease virus into Eastern Europe. *Transboundary and emerging diseases* **64**, 1001-1007 (2017).

14 Zhu, J. *et al.* Characterization of virulent Newcastle disease viruses from vaccinated chicken flocks in Eastern China. *BMC veterinary research* **12**, 113 (2016).

15 Jeon, W.-J. *et al.* Protective efficacy of commercial inactivated Newcastle disease virus vaccines in chickens against a recent Korean epizootic strain. *Journal of veterinary science* **9**, 295-300 (2008).

16 Goldhaft, T. M. Guest editorial: Historical note on the origin of the LaSota strain of Newcastle disease virus. *Avian diseases* **24**, 297-301 (1980).

17 Kapczynski, D. R. & King, D. J. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* **23**, 3424-3433 (2005).

18 Yang, H.-m., Zhao, J., Xue, J., Yang, Y.-l. & Zhang, G.-z. Antigenic variation of LaSota and genotype VII Newcastle disease virus (NDV) and their efficacy against challenge with velogenic NDV. *Vaccine* **35**, 27-32 (2017).

19 Hu, Z. *et al.* Generation of a genotype VII Newcastle disease virus vaccine candidate with high yield in embryonated chicken eggs. *Avian diseases* **55**, 391-397 (2011).

20 Miller, P. J. *et al.* Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. *Developmental & Comparative Immunology* **41**, 505-513 (2013).

21 Miller, P. J., King, D. J., Afonso, C. L. & Suarez, D. L. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. *Vaccine* **25**, 7238-7246 (2007).

22 Farhid Hemmatzadeh, M. M. A., Esmaeil Ebrahimie, Simson Tarigan, Mohammad Indro Cahyono. Molecular characterisation of newly emerged Newcastle disease viruses in Indonesia (Australian Centre for International Agricultural Research (ACIAR), Canbera, Australia, 2016).

23 Miller, P., Koch, G. & Suarez, D. L. in *Diseases of poultry* (eds David E. Swayne & John R. Glisson) 89-138 (Wiley-Blackwell, 2013).

24 Maas, R., Oei, H., Kemper, S., Koch, G. & Visser, L. The use of homologous virus in the haemagglutination-inhibition assay after vaccination with Newcastle disease virus strain La Sota or Clone30 leads to an over estimation of protective serum antibody titres. *Avian pathology* **27**, 625-631 (1998).

25 De Wit, J., Van De Sande, H., Counotte, G. & Wellenberg, G. Analyses of the results of different test systems in the 2005 global proficiency testing schemes for infectious bursal disease virus and Newcastle disease virus antibody detection in chicken serum. *Avian pathology* **36**, 177-183 (2007).

26 Panda, A., Huang, Z., Elankumaran, S., Rockemann, D. D. & Samal, S. K. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microbial pathogenesis* **36**, 1-10 (2004).

27 Choi, K.-S., Lee, E.-K., Jeon, W.-J. & Kwon, J.-H. Antigenic and immunogenic investigation of the virulence motif of the Newcastle disease virus fusion protein. *Journal of veterinary science* **11**, 205-211 (2010).

28 Doan, P. T. K. *et al.* Genome Sequences of Newcastle Disease Virus Strains from Two Outbreaks in Indonesia. *Microbiology Resource Announcements* **9** (2020).

29 Rabiei, M. *et al.* Genome Sequences of Newly Emerged Newcastle Disease Virus Strains Isolated from Disease Outbreaks in Indonesia. *Microbiology Resource Announcements* **9** (2020).

30 in *World Animal Health - 2012. Volume 1* (OIE (World Organisation for Animal Health), Paris; France, 2013).

31 Ramakrishnan, M. A. Determination of 50% endpoint titer using a simple formula. *World journal of virology* **5**, 85-86, doi:10.5501/wjv.v5.i2.85 (2016).

32 (FAO), F. a. A. O. *A Basic Laboratory Manual for the Small-Scale Production and Testing of I-2 Newcastle Disease Vaccine*, <http://www.fao.org/docrep/005/ac802e/ac802e00.htm#Contents> (2002).

33 Mahboob, T., M, A., H, A. & Siddique, M. *Preparation and Evaluation of Newcastle Disease Oil Emulsion Vaccine at Hydrophile Lipophile Balance 7.0 Using Mukteswar Strain*. (1999).

34 Reddy GS, S. V. Comparison of two experimental binary ethylenimine (BEI) inactivated Newcastle disease oil-emulsion vaccines. *Acta virologica* **35**, 3 (1991).

35 OIE. 964-983 (OIE, 2019).

36 Hemmatzadeh, F. *et al.* Recombinant M2e protein-based ELISA: a novel and inexpensive approach for differentiating avian influenza infected chickens from vaccinated ones. *PloS one* **8**, e56801 (2013).

37 Hemmatzadeh, F., Niap, F., Bennett, B. A., Trott, D. J. & Peaston, A. E. A novel quantitative polymerase chain reaction to monitor urinary tract mycoplasma infection in a dog. *Letters in applied microbiology* **68**, 409-414 (2019).

38 Lee, C., Kim, J., Shin, S. G. & Hwang, S. Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. *Journal of biotechnology* **123**, 273-280 (2006).

39 Shirima, R. R. *et al.* Absolute quantification of cassava brown streak virus mRNA by real-time qPCR. *Journal of Virological Methods* **245**, 5-13, doi:https://doi.org/10.1016/j.jviromet.2017.03.003 (2017).

40 Xiao, S. *et al.* (Am Soc Microbiol, 2012).

41 Li, T. *et al.* Comprehensive analysis and characterization of linear antigenic domains on HN protein from genotype VII Newcastle disease virus using yeast surface display system. *Plos one* **10**, e0131723 (2015).

42 Liu, J. *et al.* Effects of the HN antigenic difference between the vaccine strain and the challenge strain of Newcastle disease virus on virus shedding and transmission. *Viruses* **9**, 225 (2017).

43 Alamares, J. G., Li, J. & Iorio, R. M. Monoclonal antibody routinely used to identify avirulent strains of Newcastle disease virus binds to an epitope at the carboxy terminus of the hemagglutinin-neuraminidase protein and recognizes individual mesogenic and velogenic strains. *Journal of clinical microbiology* **43**, 4229 (2005).

44 Cosset, F.-l. *et al.* Newcastle disease virus (NDV) vaccine based on immunization with avian cells expressing the NDV hemagglutinin-beuraminidase glycoprotein. *Virology* **185**, 862-866 (1991).

45 Yusoff, K., Nesbit, M., McCartney, H., Emmerson, P. T. & Samson, A. C. Mapping of three antigenic sites on the haemagglutinin-neuraminidase protein of Newcastle disease virus. *Virus research* **11**, 319-333 (1988).

46 Iorio, R. M. *et al.* Neutralization map of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition. *Journal of virology* **65**, 4999-5006 (1991).

47 Iorio, R. M., Glickman, R. L., Riel, A. M., Sheehan, J. P. & Bratt, M. A. Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. *Virus research* **13**, 245-261 (1989).

48 Hu, S. *et al.* Identification of a variable epitope on the Newcastle disease virus hemagglutinin–neuraminidase protein. *Veterinary microbiology* **140**, 92-97 (2010).

49 Iorio, R. M., Lawton, K. A., Nicholson, P. M. & Bratt, M. A. Monoclonal antibodies identify a strain-specific epitope on the HN glycoprotein of Newcastle disease virus strain Australia-Victoria. *Virus research* **1**, 513-525 (1984).

50 Susta, L. *et al.* Pathologic characterization of genotypes XIV and XVII Newcastle disease viruses and efficacy of classical vaccination on specific pathogen-free birds. *Veterinary pathology* **52**, 120-131 (2015).

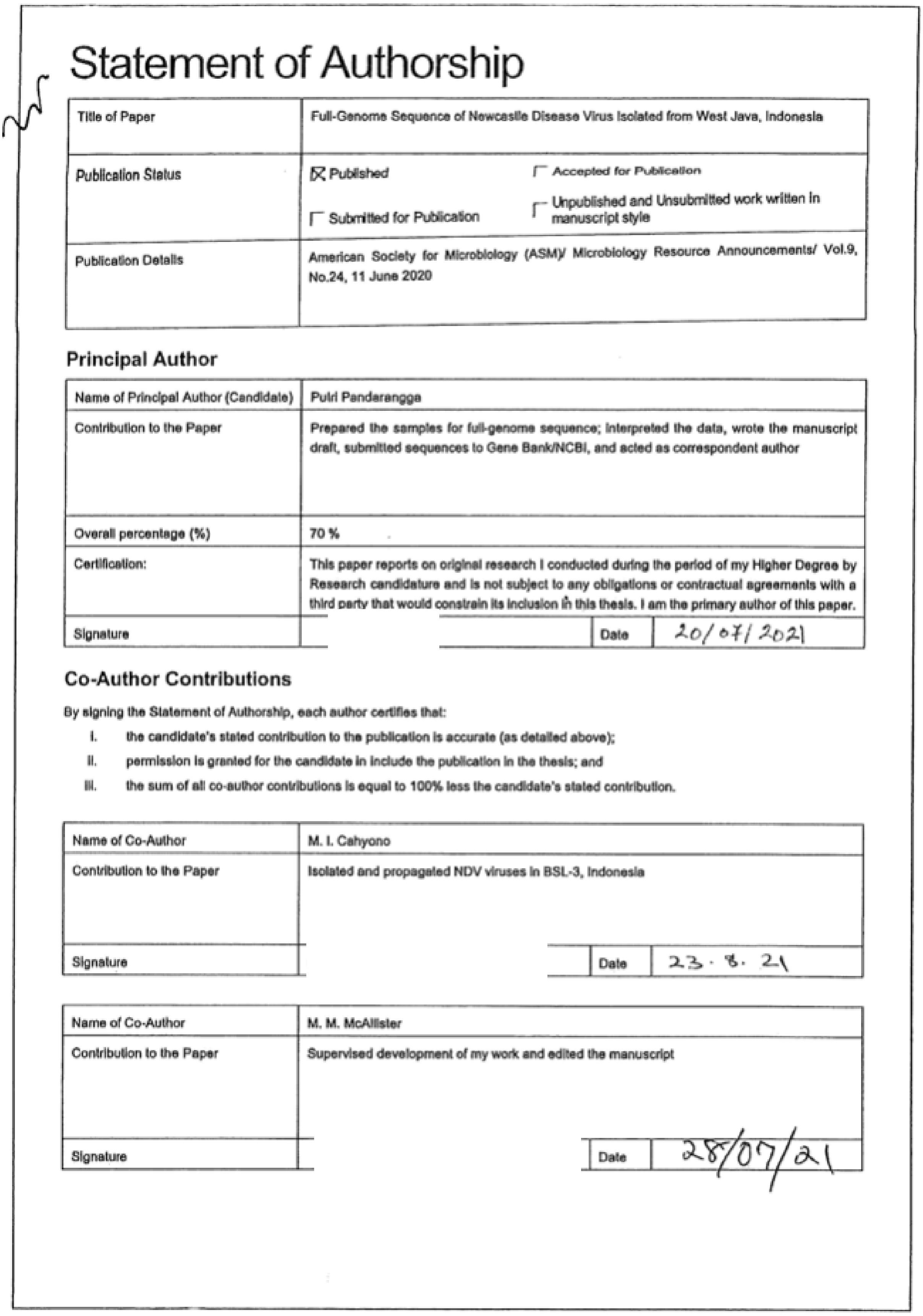
51 de Leeuw, O. S., Koch, G., Hartog, L., Ravenshorst, N. & Peeters, B. P. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin–neuraminidase protein. *Journal of General Virology* **86**, 1759-1769 (2005).

52 Tan, S. W., Ideris, A., Omar, A. R., Yusoff, K. & Hair-Bejo, M. Sequence and phylogenetic analysis of Newcastle disease virus genotypes isolated in Malaysia between 2004 and 2005. *Archives of virology* **155**, 63-70 (2010).

53 Abolnik, C. & Wandrag, D. B. Avian gyrovirus 2 and avirulent Newcastle disease virus coinfection in a chicken flock with neurologic symptoms and high mortalities. *Avian diseases* **58**, 90-94 (2014).

CHAPTER 4

FULL-GENOME SEQUENCE OF NEWCASTLE DISEASE VIRUS ISOLATED FROM WEST JAVA, INDONESIA



Statement of Authorship

Title of Paper	Full-Genome Sequence of Newcastle Disease Virus Isolated from West Java, Indonesia
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	American Society for Microbiology (ASM)/ Microbiology Resource Announcements/ Vol.9, No.24, 11 June 2020

Principal Author

Name of Principal Author (Candidate)	Putri Pandarangga		
Contribution to the Paper	Prepared the samples for full-genome sequence; interpreted the data, wrote the manuscript draft, submitted sequences to Gene Bank/NCBI, and acted as correspondent author		
Overall percentage (%)	70 %		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	20/07/2021

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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Contribution to the Paper	Isolated and propagated NDV viruses in BSL-3, Indonesia		
Signature		Date	23 . 8 . 21

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Contribution to the Paper	Supervised development of my work and edited the manuscript		
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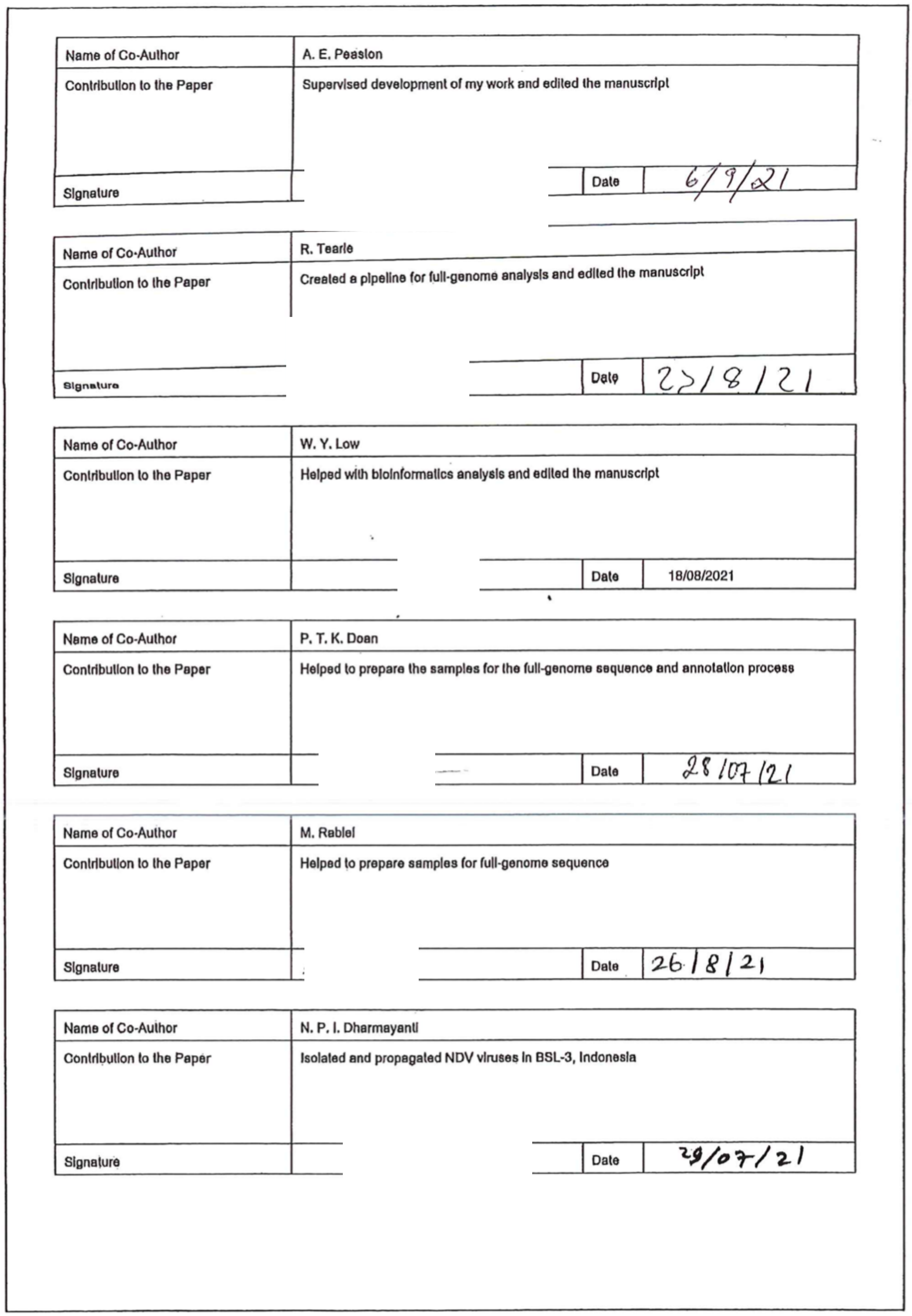

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Name of Co-Author	P. T. K. Doan		
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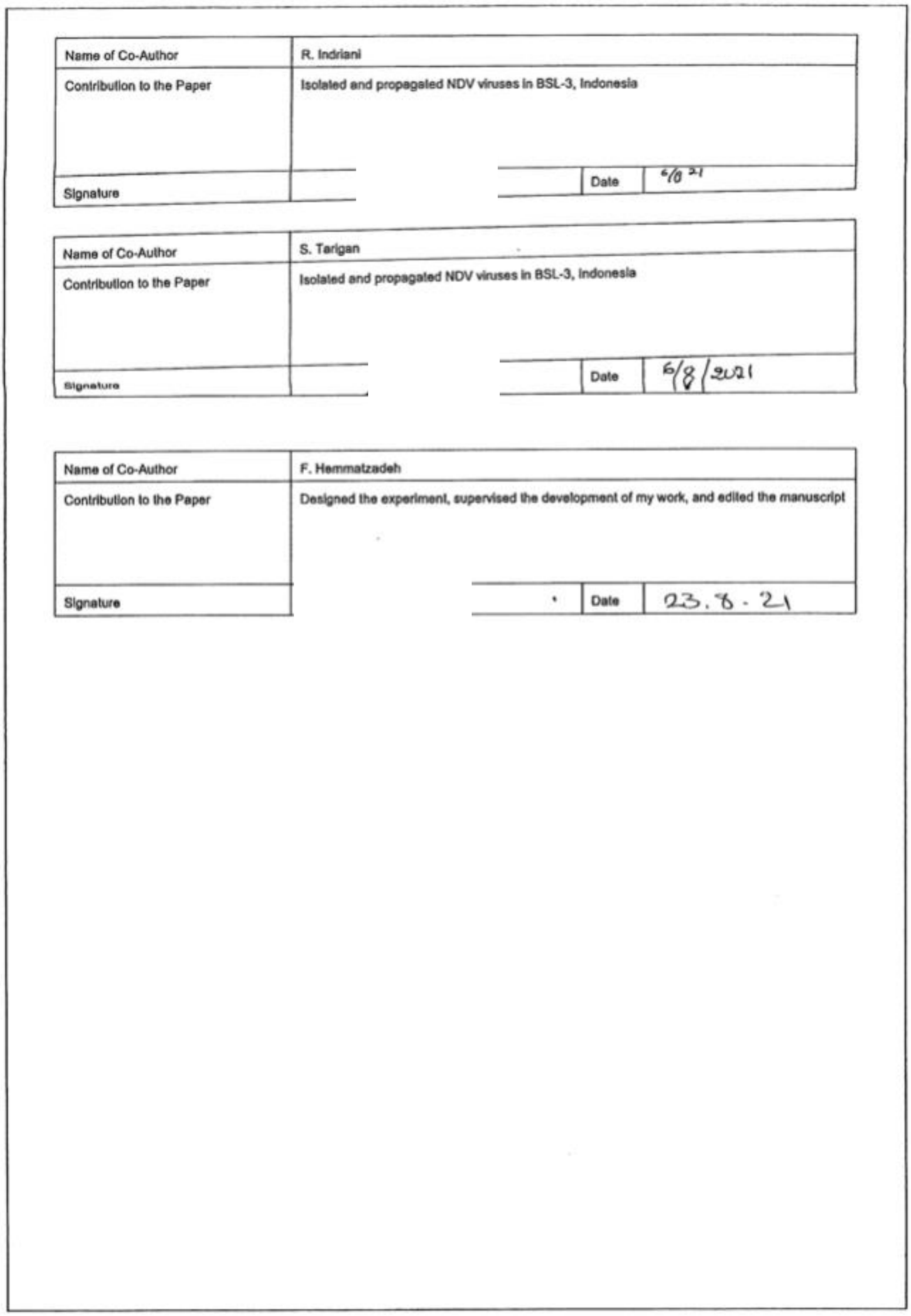

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Full-Genome Sequence of Newcastle Disease Virus Isolated from West Java, Indonesia

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- The manuscript had been published in **Microbiology Resources Announcements Journal**. However, due to the journal's requirement, the content is no more than one page, so that some information is not included in the journal. Hence, additional data and formatting are modified in this chapter.
- The phylogeny tree had been presented to **the Fifth Symposium of the Association of Japan-Indonesia Veterinary Education 2021** (13th February 2021).

Abstract

The full-genome sequence was examined for chicken/Indonesia/Cilebut/010WJ/2015 and chicken/Indonesia/ITA/012WJ/1951 strains isolated from West Java, Indonesia in 2015 and 1951, respectively. Chicken/Indonesia/Cilebut/010WJ/2015 (Genotype VII) caused a 2015 disease outbreak in Indonesia, and chicken/Indonesia/ITA/012WJ/1951 (Genotype VI) is used as a standard strain for the challenge in NDV vaccine trials. Based on the phylogeny tree showed genotype differences between the vaccine, a standard challenge and NDV strains circulating is likely caused NDV outbreaks in Indonesia.

Keywords: Newcastle Disease Virus; Full Genome Sequence; Virology

4. 1. Introduction

In Indonesia, Newcastle disease (ND) outbreaks have caused high mortality in commercial chickens, even those vaccinated with attenuated Newcastle Disease Virus (NDV) [1]. Formally, NDV is *Avian Orthoavulavirus* 1 and type virus of the *Orthoavulavirus* genus, *Paramyxoviridae* family [2]. NDV is divided into two classes based on the F gene sequence, namely classes I and II. Class II has 21 genotypes with varying virulence. GVII NDV predominates in Southeast Asia [3,4], but local vaccines mostly contain GII strain LaSota [5].

GVII has spread almost throughout Indonesia, including in the largest islands of Indonesia such as Java, Borneo (Kalimantan), and the Papuan islands [5]. However, local vaccines mostly contain GII strain LaSota. Other strains collected in Indonesia are Chicken/Indonesia/Cilebut/010WJ/2015 (Cilebut) and chicken/Indonesia/ITA/012WJ/1951 (ITA), which are virulent strains [5]. Strain Cilebut was collected in 2015 from chicken brains in West Java. Strain ITA was collected in West Java, Indonesia, in 1951 and today is used in challenge trials to test the efficacy of vaccines [5]. However, these two strains have never been fully sequenced.

We hypothesize that antigenic mismatch between GVII and strain ITA used in challenge trials has caused unreliability in vaccine challenge test results, enabling ND outbreaks in vaccinated chickens. Therefore, the purpose of this study was to investigate the similarity of the two strains at the sequence level. A phylogenetic tree approach was also taken to compare the two strains above, vaccines, and other strains circulating in Indonesia.

4. 2. Materials and Methods

4. 2. 1. Newcastle Disease Virus Isolation

The strain chicken/Indonesia/Cilebut/010WJ/2015 was isolated from the 2015 NDV outbreak in Cilebut, West Java, Indonesia, while the strain chicken/Indonesia/ITA/012WJ/1951 strain was derived from the *Balai Besar Penelitian Veteriner* (BALITVET) repository in Indonesia. This latter strain is regularly used to determine the effectiveness of any new NDV vaccine in Indonesia. Both strains were propagated in allantoic cavities of 9-day-old SPF chicken eggs, with fluid was collected by day four or the moment the embryo dies [6]. This presence of virus was confirmed using a hemagglutination (HA) test [7].

4. 2. 2. Viral RNA Extraction

Viral RNA was extracted from the allantoic fluid using RNeasy Plus Universal Kits (Qiagen) following the manufacturer’s protocol. The concentration and quality of viral RNA were measured using a 2200 TapeStation system (Agilent Technologies, USA). Complementary DNA (cDNA) libraries were synthesized using a Kapa-stranded RNA library kit based on the manufacturer's protocol. cDNA was sequenced on an Illumina MiSeq instrument to generate 2 x 300-nucleotide (nt) reads.

4. **2. 3. Data Analysis**

Raw sequence data were assembled using Unicycler V0.4.8 with default parameters [8]. Assembled scaffolds were extracted using BANDAGE V0.8.1 [9]. All the contigs were compared to existing NDV sequences using the Basic Local Alignment Search Tool (BLAST). Minimap2 V2.17 was used to align and determine the orientation of contigs to the existing NDV sequence [10]. In this way, a relatively complete genome was reconstructed with contigs matching the consensus order. The sequence was compared to all known sequences using BLAST with nr/nt databases. Finally, the genome was annotated using Geneious V2020.0.3.

4. 2. 4. Phylogenetic Tree Construction

Not all NCBI NDV sequences from Indonesia were used to construct a phylogeny tree. Only one sequence from each province in Indonesia was used to see the distribution of genotype VII. All NDV sequences were downloaded from NCBI except the ITA and Cilebut strains. All the sequences were aligned using BioEdit software. Evolutionary analyses were conducted using MEGA X [11]. The evolutionary history was inferred using the Neighbor-Joining method [12]. The optimal tree is shown (Figure 4. 3). The percentage of replicate trees in which the associated taxa clustered together in a bootstrap test (2000 replicates) is shown next to the branches [13]. The evolutionary distances were computed using the Maximum Composite Likelihood method [14] and are in the units of the number of base substitutions per site.

4. 3. Result and Discussion

This study showed that the strain isolated from the outbreak in Cilebut, West Java, Indonesia, in 2015 was of GVII. This finding strengthens the overall finding that GVII is the dominant genotype causing NDV outbreaks across Indonesia and is consistent with the phylogenic tree (Figure 4. 3). In comparison, the ITA strain, first isolated in 1951, belongs to GVI.

4. **3. 1. Chicken/Indonesia/ITA/012WJ/1951**

Assembly of the ITA isolate by Unicycler used 636,469 paired reads as input. Three contigs formed a scaffold that shows sequence similarity to NDV: contig 2 (length 14,539 bp and coverage 246.87), contig 160 (length 7bp and coverage 75.91), and contig 152 (length 823bp and coverage, 175.8) – see Figure 4. 1.

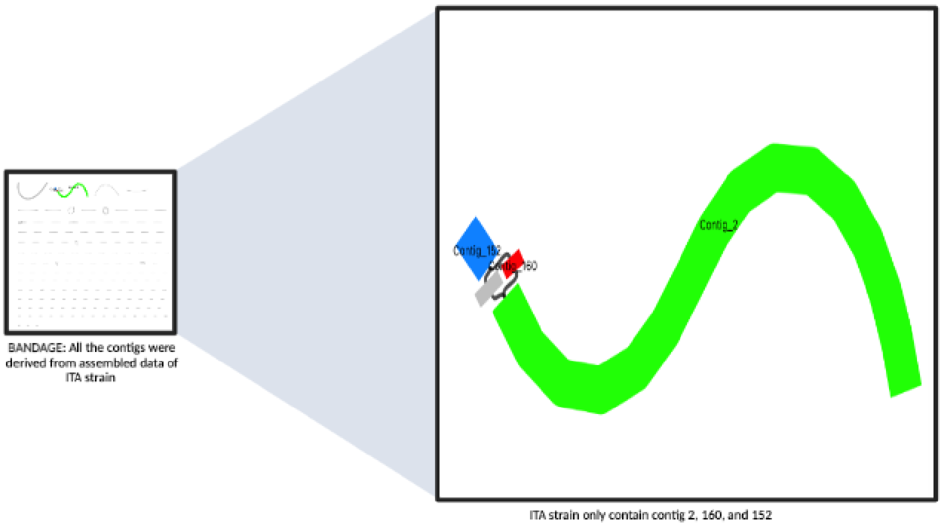

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Figure 4. 1. Contigs of ITA strain that extracted using BANDAGE. Total contigs are extracted from assembled data of ITA strain were 161 contigs (left square). However, only contig 2 (green), contig 160 (red), and contig 152 (blue) map to NDV.

These contigs were compared to existing NDV sequences using BLAST and showed 94 to 96% identity with chicken/U.S.(CA)/1083(Fontana)/72 (Fontana; GenBank accession number AY562988.1). These contigs were also aligned to strain Fontana using minimap2 V2.17 to determine their orientation. The 3 contigs aligned contiguously and without gaps or ambiguity to the reference sequence of strain Fontana as follows: contig 152, from 102 to 822 bp; contig 160, from 823 to 829 bp; and contig 2, from 830 to 15,369 bp. This suggests a complete genome sequence could be reconstructed by concatenating the three contigs in the appropriate order. The final genome of strain ITA had a total length of 15,369 bp with 1,372-fold coverage, and GC content of 46.0%. The sequence was compared to all known sequences using BLAST and the NCBI nonredundant/nucleotide (nr/nt) databases. The top BLAST hit showed 94.7% identity with strain Fontana. The genome was annotated using Geneious

V2020.0.3. Sequence alignment and phylogenetic analysis (Figure 3) confirmed that strain ITA belongs to genotype VI with ^{112}R-R-Q-K-R-F^{117} as the cleavage site motif of the fusion protein.

4. 3. 2. Chicken/Indonesia/Cilebut/010WJ/2015

The Cilebut strain was similarly assembled using 989,005 paired reads. A single NDV contig was assembled with a total length of 15,225 bp, 246-fold coverage (Figure 4. 2), and a GC content of 46.2%. The top BLAST hit showed 97.07% identity with NDV isolate IBS005/11 (GenBank accession number KR074405.1). Annotation was performed as described above. Sequence alignment and phylogenetic analysis (Figure 4. 3) of strain Cilebut revealed that it belongs to genotype VII, with the fusion protein cleavage site motif ^{112}R-R-Q-K-R-F^{117}.

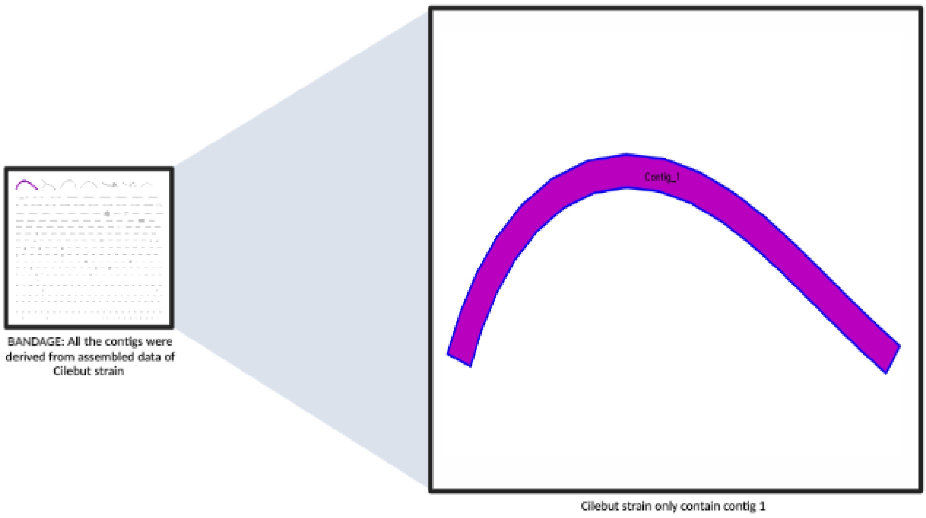

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Figure 4. 2. Contigs of Cilebut strain that extracted using BANDAGE. Total contigs are extracted from assembled data of ITA strain was 431 contigs (left square). However, only contig 1 (purple) build the chicken/Indonesia/Cilebut/010WJ/2015.

4. 3. 3. An NDV Phylogeny Tree

The two new sequences reported here were combined with 37 existing sequences to construct a phylogeny tree (Figure 4. 3). The phylogeny analysis shows that the GVII genotype is the dominant genotype causing outbreaks throughout Indonesia, from Sumatra to Papua (Figure 4. 4). However, the most used vaccine belongs to GII.

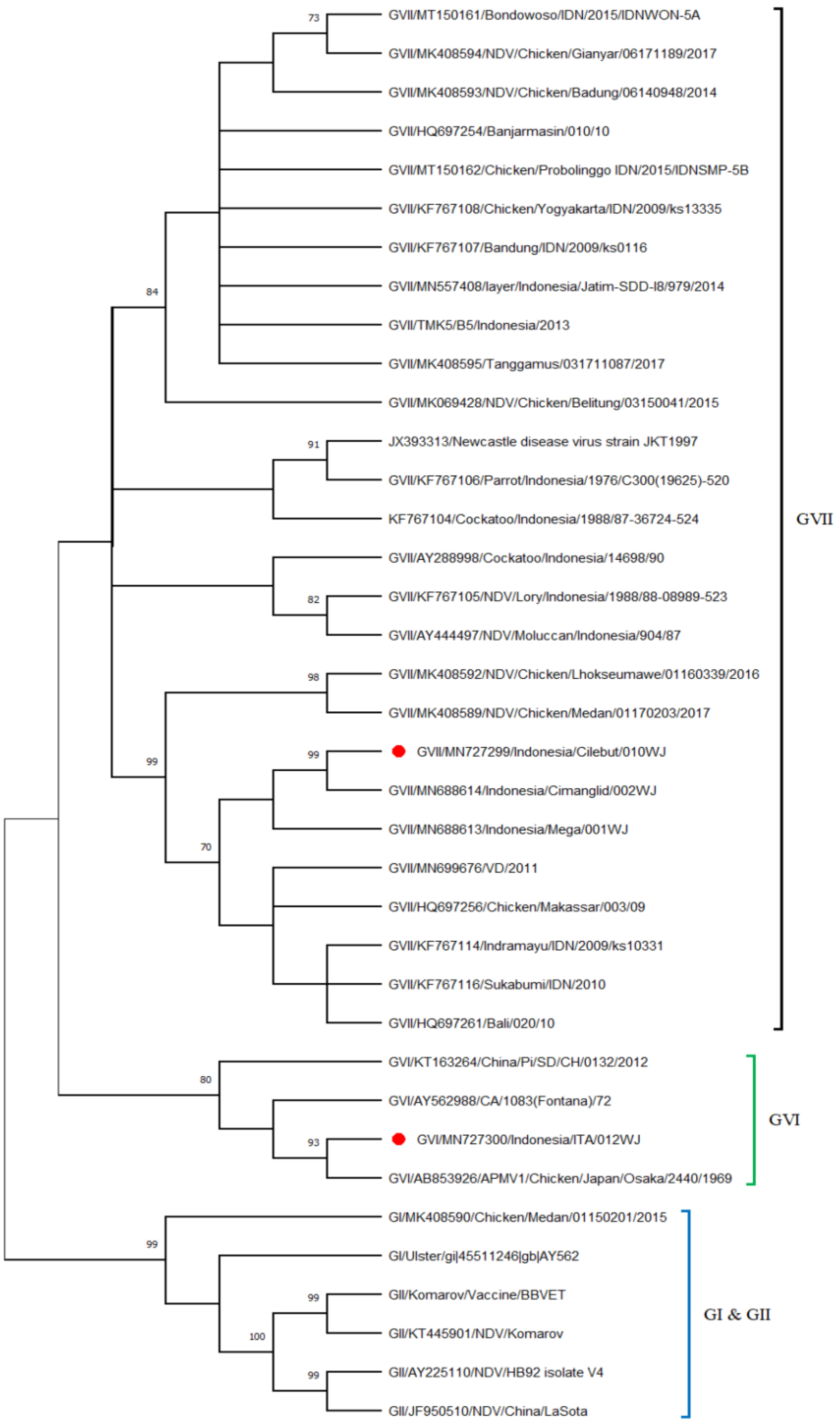

Figure 4. 3. Phylogeny tree of NDV in Indonesia. This analysis was conducted with MEGA X using 37 nucleotide sequences. Evolutionary history and distances were computed using the Neighbor-Joining method and Maximum Composite Likelihood method, respectively. Our NDV sequences were marked with a red circle. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 408 positions in the final dataset.

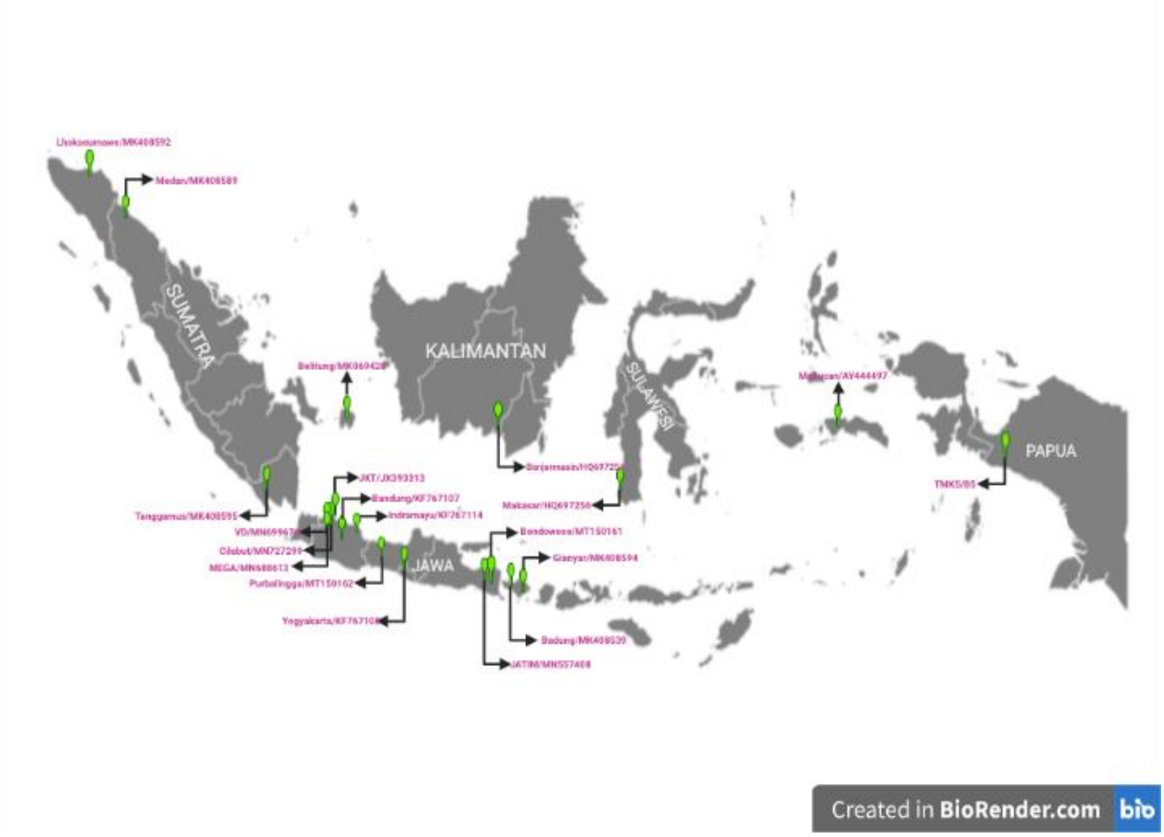

Figure 4. 4. The distribution of genotype VII causes NDV outbreaks throughout Indonesia.

In conclusion, GVII NDVs are predominantly responsible for ND outbreaks in Indonesia. However, GII is used for most Indonesian vaccines, and the standard challenge strain used in vaccine efficacy trials is from GVI. Thus, antigenic disparities between the vaccine, challenge, and field strains of NDV may enable disease outbreaks in vaccinated chickens.

4. 4. Nucleotide Sequence Accession Numbers

The GenBank and the Sequence Read Archive (SRA) accession numbers for chicken/Indonesia/Cilebut/010WJ/2015 are MN727299 and SRR11593163, whilst chicken/Indonesia/ITA/012WJ/1951 are MN727300 and SRR11593165, respectively.

4. 5. Acknowledgments

The work was supported by The Australian Centre for International Agricultural Research (ACIAR) under the AH/2015/003 and AH/2010/039 research projects.

4. 6. References

1 Xiao, S. *et al.* (Am Soc Microbiol, 2012).

2 Rima, B. *et al.* ICTV Virus Taxonomy Profile: Paramyxoviridae. *Journal of General Virology*, jgv001328 (2019).

3 Tan, S. W., Ideris, A., Omar, A. R., Yusoff, K. & Hair-Bejo, M. Sequence and phylogenetic analysis of Newcastle disease virus genotypes isolated in Malaysia between 2004 and 2005. *Archives of virology* **155**, 63-70 (2010).

4 Choi, K.-S. *et al.* Molecular epidemiology of Newcastle disease viruses in Vietnam. *Tropical animal health and production* **46**, 271-277 (2014).

5 Farhid Hemmatzadeh, M. M. A., Esmaeil Ebrahimie, Simson Tarigan, Mohammad Indro Cahyono. Molecular characterisation of newly emerged Newcastle disease viruses in Indonesia (Australian Centre for International Agricultural Research (ACIAR), Canbera, Australia, 2016).

6 Diel, D. G. *et al.* Complete genome and clinicopathological characterization of a virulent Newcastle disease virus isolate from South America. *Journal of clinical microbiology* **50**, 378-387 (2012).

7 OIE. 964-983 (OIE, 2019).

8 Wick, R. R., Judd, L. M., Gorrie, C. L. & Holt, K. E. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS computational biology* **13**, e1005595 (2017).

9 Wick, R. R., Schultz, M. B., Zobel, J. & Holt, K. E. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* **31**, 3350-3352 (2015).

10 Li, H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* **34**, 3094-3100 (2018).

11 Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution* **35**, 1547 (2018).

12 Saitou, N. & Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution* **4**, 406-425 (1987).

13 Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. *evolution* **39**, 783-791 (1985).

14 Tamura, K., Nei, M. & Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* **101**, 11030-11035 (2004).

CHAPTER 5

GENERAL DISCUSSION

5. 1. General Discussion

Overall, this study was conducted to investigate differences in the immune response of chickens to the homologous vaccine (GVII vaccine) and the commercial vaccine (GII vaccine). The differences were determined at the molecular level using the spleen transcriptomic method, serology tests, and viral loading counting. In addition, with a whole-genome sequence, it was determined that genotype VII was the dominant strain causing outbreaks in Indonesia.

The literature review investigation mainly emphasizes the host's reaction to vaccines adaptive immunity, which consists of cell-mediated immunity and humoral immunity as antibody-producing. Moreover, antibody production is still a key modulator of protection in the host, but CMI also reduces viral shedding [1]. However, as technology develops, knowledge also increases that the process and network of host gene interactions in response to vaccines are complex, so a biological system approach is needed [2,3]. This approach provides a more comprehensive explanation than methods that only isolate and characterize several components of the host reaction to the vaccine. For NDV vaccines, the development is quite fast from 1950 until now [4-6]. However, the efficacy of NDV vaccines is only tested using a few components of the chicken response to the NDV vaccine at the molecular level, and the standard method is used until now [5,7-9].

Based on the reasons above, the first objective of this study was to determine the difference in the immune response of chickens after being vaccinated using the GVII vaccine and the GII vaccine at the gene level. Spleen transcriptomic as part of the system biology approach was applied to determine the interaction between host genes and form a network in protecting chickens after being vaccinated. Surprisingly, unlike the findings of most vaccines, the predicted canonical pathway of homologous vaccines is related to down-regulated neuroinflammation and up-regulated synaptogenesis. We speculated that the down-regulated immune system regulation protected the nervous system from excess leukocytes and cytokine activity. Previous studies have proven that NF-B is a central component that can protect the neuron system if it is down-regulated [10-12]. In contrast, up-regulation of synaptogenesis allows neurons to communicate with each other to maintain homeostasis [13]. CMI, part of adaptive immunity, is also seen in the prediction list but is not included in canonical pathways. At the same time, the commercial vaccine stimulates downregulated the unfolded protein response (UPR), which is associated with inhibiting apoptosis. However, the software's drawback (Ingenuity Pathway Analysis/IPA) is predicting the results from existing knowledge, namely mice and humans. Thus, caution is needed in the interpretation of the results from the chicken data set.

In the second objective, evidence is also carried out at serology and viral loading counting. The homolog vaccine (GVII) provides better protection by significantly reducing viral shedding after being challenged with NDV GVII, currently circulating in Indonesia. Other results obtained from the HI test, sera of vaccinated chicken with the homologous vaccine have higher antibody titers when tested with homologous antigens. Moreover, chickens vaccinated with the homologous vaccine (GVII) when challenged with GVII could decrease viral shedding. The results of this test are a recommendation for veterinary laboratories in Indonesia that the use of homologous antigens in the field provides relevant antibody titers compared to the use of heterologous antigens, namely RIVS. Also, in this study, alternative protein peptides from the cleavage site of F protein from GII and GVII were tested as a substitute for the whole virus antigen. However, the results did not support our hypothesis that the cleavage site of F protein can be an alternative antigen in the ELISA test.

Finally, to prove that genotype VII is still the genotype that causes outbreaks in Indonesia, a whole-genome sequence was performed for one of the outbreaks in West Java, Indonesia. In addition, we hypothesize that the vaccine, antigen, and challenge ND virus must be homologous to the genotype that causes the outbreaks in the field. Hence, the challenge strain, namely ITA, in determining vaccine efficacy in Indonesia is carried out in a whole genome sequence. This virus challenge has been used as a national standard for determining vaccine efficacy in Indonesia. Furthermore, the phylogenetic tree results showed that the strain causing the outbreak came from GVII while the challenge virus belongs to GVI. Therefore, in future research, other proteins in NDV are needed to evaluate the different immunological domains as candidate of new antigens in serological tests.

5. 2. Conclusion

In conclusion, based on the results of this study, three recommendations can be given. First, to know the responses of chickens to the new NDV vaccine, a system biology approach should be used to determine the molecular signature as its hallmark. Second, due to antigenic disparities between the vaccine, challenge, and field strains of NDV, all genotypes used must be the same as those in the field. Third, the use of RIVS as a tested antigen and ITA as a challenge virus needs to be considered at the veterinary lab in Indonesia to obtain relevant antibodies and determine the efficacy of a new vaccine, respectively.

5. 3. Reference

1 Russell, P., Dwivedi, P. & Davison, T. The effects of cyclosporin A and cyclophosphamide on the populations of B and T cells and virus in the Harderian gland of chickens vaccinated with the Hitchner B1 strain of Newcastle disease virus. *Veterinary immunology and immunopathology* **60**, 171-185 (1997).

2 Pezeshki, A., Ovsyannikova, I. G., McKinney, B. A., Poland, G. A. & Kennedy, R. B. The role of systems biology approaches in determining molecular signatures for the development of more effective vaccines. *Expert review of vaccines* **18**, 253-267 (2019).

3 Pulendran, B., Li, S. & Nakaya, H. I. Systems vaccinology. *Immunity* **33**, 516-529 (2010).

4 Basavarajappa, M. K. *et al.* A recombinant Newcastle disease virus (NDV) expressing infectious laryngotracheitis virus (ILTV) surface glycoprotein D protects against highly virulent ILTV and NDV challenges in chickens. *Vaccine* **32**, 3555-3563 (2014).

5 Ji, Y. *et al.* A novel genotype VII Newcastle disease virus vaccine candidate generated by mutation in the L and F genes confers improved protection in chickens. *Veterinary microbiology* **216**, 99-106 (2018).

6 Liu, M.-M. *et al.* Generation by reverse genetics of an effective attenuated Newcastle disease virus vaccine based on a prevalent highly virulent Chinese strain. *Biotechnology letters* **37**, 1287-1296 (2015).

7 Nikbakht Brujeni, G., Hassanzadeh, M., Al-Karagoly, H., Tolouei, T. & Esmailnejad, A. Evaluation of humoral immune responses to enterotropic lentogenic VG/GA vaccine of Newcastle disease in commercial turkey poults (Meleagris gallopavo). *Acta Veterinaria Scandinavica* **61**, 1-6 (2019).

8 Lambrecht, B., Gonze, M., Meulemans, G. & van denberg, T. P. Assessment of the cell-mediated immune response in chickens by detection of chicken interferon-γ in response to mitogen and recall Newcastle disease viral antigen stimulation. *Avian Pathology* **33**, 343-350 (2004).

9 Sedeik, M. *et al.* Comparative efficacy of commercial inactivated Newcastle disease virus vaccines against Newcastle disease virus genotype VII in broiler chickens. *Poultry science* **98**, 2000-2007 (2019).

10 Brambilla, R. *et al.* Transgenic inhibition of astroglial NF-κB protects from optic nerve damage and retinal ganglion cell loss in experimental optic neuritis. *Journal of neuroinflammation* **9**, 1-12 (2012).

11 Brambilla, R. *et al.* Transgenic inhibition of astroglial NF-κB improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation. *The Journal of Immunology* **182**, 2628-2640 (2009).

12 Liot, G. *et al.* Neurotrophin-3-induced PI-3 kinase/Akt signaling rescues cortical neurons from apoptosis. *Experimental neurology* **187**, 38-46 (2004).

13 Nisticò, R. *et al.* Synaptoimmunology-roles in health and disease. *Molecular brain* **10**, 1-12 (2017).