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Original article

IMMUNOINFORMATIC ANALYSIS OF HAEMAGGLUTININ-NEURAMINIDASE PROTEIN TO REVEAL MOLECULAR IMMUNOGENICITY OF NEWCASTLE DISEASE VIRUS BETWEEN VACCINE AND INDONESIAN STRAIN

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Summary

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Newcastle disease (ND) is a significant avian disease in Indonesia, causing substantial economic losses in poultry production. Despite vaccination efforts, ND outbreaks persist, raising questions about vaccine efficacy. This study investigates the molecular immunogenicity of the haemagglutinin-neuraminidase (HN) protein between the widely used Lasota vaccine strain and Indonesian Newcastle disease virus (NDV) strains. Phylogenetic analysis revealed two main clusters, with the Lasota strain showing 86.1–99.8% identity to Indonesian strains. Secondary structure analysis and epitope predictions highlighted key antigenic differences, including mutations in critical epitopes. Only 8.9% of Lasota's predicted B- and T-cell epitopes were conserved in Indonesian strains, suggesting limited cross-protection. The findings suggest that molecular variations in the HN protein may contribute to ND recurrence in vaccinated flocks by allowing immune evasion. This emphasises the need for vaccines tailored to the molecular characteristics of circulating strains. By leveraging immunoinformatic tools, this study provides insights into the design of improved vaccines that target both humoral and cellular immune responses. In conclusion, significant differences in immunogenicity between the Lasota and Indonesian NDV strains underline the importance of updating vaccine strategies. These findings are critical for ND control in Indonesia and have broader implications for global vaccine development against evolving NDV strains.

Key words: haemagglutinin-neuraminidase, immunoinformatic, molecular immunogenicity, Newcastle disease virus, poultry disease

INTRODUCTION

Newcastle disease (ND) is a widespread avian disease in Indonesia that causes interruptions in chicken production. The widespread distribution of this respiratory virus poses a huge challenge to the poultry sector, leading to substantial economic impacts (Swayne *et al.,* 2020; Yehia *et al.,* 2023). The virus (NDV) that causes the illness originates from the taxon genus *Orthoavulavirus*, which is a member of the family *Paramyxoviridae's* subfamily *Avulavirinae*, according to ICTV (Lefkowitz *et al.,* 2018; Amarasinghe *et al.,* 2019; Rima *et al.,* 2019). This disease is endemic in Indonesia and usually caused an outbreak (Doan *et al.,* 2020; Rabiei *et al.,* 2020). Even among birds that have received all vaccinations, the ND virus can infect different kinds of Indonesia fowl (Doan *et al.,* 2020; Pandarangga *et al.,* 2020). Surprisingly, ND is also known to infect pigeons (Dharmayanti *et al.,* 2014; Pestka *et al.,* 2014; Dewandaru *et al.,* 2020; Andriyani *et al.,* 2022; Sheng *et al.,* 2024), turtledoves (Andriyani *et al.,* 2022), peacocks (Angeliya *et al.,* 2023), and eagles (Angeliya *et al.,* 2022a) in addition to mainstream commercial poultry as reported previously (Goraichuk *et al.,* 2020; Angeliya *et al.,* 2022b; Giang *et al.,* 2023; Kamal *et al.,* 2024).

The molecular characteristics of the ND virus are thought to have an impact on the frequently reported disruption in poultry productivity. NDV is an enclosed virus with a single-stranded RNA genome that is negative-sense and non-segmented. Six key structural proteins are encoded by the genome: large polymerase protein (L), fusion protein (F), matrix protein (M), phosphoprotein (P), nucleoprotein (NP), and haemagglutinin-neuraminidase (HN) (MacLachlan & Dubovi, 2011; Rima *et al.,* 2019). The virulence and pathogenicity of the virus are determined by the F and HN proteins, which are essential for viral attachment, fusion, and entrance into host cells (MacLachlan & Dubovi, 2011; Rima *et al.,* 2019). Because the ND virus' genome is RNA-based, it lacks a proofreading system, which can facilitate the rapid mutation of viral proteins (MacLachlan & Dubovi, 2011; Simon-Loriere & Holmes, 2011). The F protein, which serves as a marker of pathogenicity (Angeliya *et al.,* 2022b; Utami *et al.,* 2023) and a determinant of the viral genotype (WOAH, 2021), also the HN protein, which serves as a measure of immunogenicity (Shafaati *et al.,* 2022; Chang *et al.,* 2023; Lu *et al.,* 2024; Sheng *et al.,* 2024) are two significant proteins impacted by mutations. For example, mutations in cleavage site of F protein have an impact on NDV pathogenicity (Ojha & Prajapati, 2021; Yehia *et al.,* 2023). HN protein mutations' effects on immunogenicity have not received much attention, and unfortunately most ND immunoinformatic research analysed only F protein (Putri *et al.,* 2021).

Despite efforts to prevent and monitor, the existence of ND cases in Indonesia remains unsatisfactorily eradicated. In the current situation of NDV in Indonesia, it is recorded that even though the vaccination program has been carried out, NDV outbreaks still occur periodically (Dharmayanti *et al.,* 2024). Vaccinated flocks can also still have cases, as mentioned previously (Doan *et al.,* 2020; Pandarangga *et al.,* 2020). Furthermore, serological diagnostic investigations have frequently been conducted for field case monitoring (Dewandaru *et al.,* 2020; Isihak *et al.,* 2020; Seung *et al.,* 2022). The commonly used serological methods for ND detection, hemagglutination-inhibition

(HI) test and enzyme linked immunosorbent assay (ELISA), are still limited to evaluating humoral immune responses as antibody titres (Isihak *et al.,* 2020; WOAH, 2021; Seung *et al.,* 2022). Unfortunately, antibody responses only are not adequate to predict the protectivity of vaccine to challenge virus (Seung *et al.,* 2022).

On the other hand, the measurement of the cellular immune response is challenging. The only available methods for testing it are expensive and less applicable, *in vivo* challenge tests (Seung *et al.,* 2022; Utami *et al.,* 2023). For instance to determine cellular immunity, some researchers use the transcriptomics approach to try and ascertain (Rabiei *et al.,* 2021), still impact on expensive cost. Another way to predict cellular immune responses is by using the in silico approach, for a wide range of pathogens, including viruses (Awadelkareem & Ali, 2020; Raoufi *et al.,* 2020; Kangabam *et al.,* 2021; Martinelli, 2022) with relatively cheaper costs.

Research on the molecular immunogenicity of NDV is important because it answers why ND cases always recur, even from well-vaccinated poultry. In contrast to other immunological assesment with challenge test methods that still require expensive costs and complicated facilities (WOAH, 2021; Seung *et al.,* 2022; Utami *et al.,* 2023), *in silico* immunological tests (immunoinformatics) (Raoufi *et al.,* 2020; Kangabam *et al.,* 2021; Martinelli, 2022; Zhu *et al.,* 2024) can be used as predictors and complements to reveal the immunogenicity properties of ND viruses, particularly in HN proteins. Unlike previous researchers who studied immunoinformatic features in the F protein (Putri *et al.,* 2021), this research focuses on another immunogenic protein, namely the HN protein. Therefore, this study aimed to carry out HN protein immunoinformatic analysis to reveal molecular immunogenicity of NDV between vaccine and Indonesian strain.

MATERIALS AND METHODS

Study design

The samples were nucleotide sequences from HN proteins from ND isolates from both vaccinated and unvaccinated flocks in Indonesia that have been genotyped based on the F protein sequence (n=23). The reference isolate was a Lasota strain, commonly used as a vaccine strain in Indonesia for both live and killed vaccines. The HN sequences of reference isolates with characterised genotypes as a point of comparison were also used.

Phylogenetic and secondary structure analysis

Phylogenetic analysis. The HN sequences were subjected to analysis, which involved predicting amino acids, performing multiple alignments, assessing polymorphism, calculating genetic distance, and constructing a phylogenetic tree using the neighbor joining method with 1000 bootstraps in MEGA-X. Amino acids were numbered based on the reference strain Lasota (de Leeuw & Peeters, 1999) from start codon (methionine).

Secondary structure analysis. The amino acid sequence from the reference strain was analysed to determine the presence of transmembrane regions and to predict its folding pattern. The Transmembrane Hidden Markov Model (TMHMM) 2.0 web server was utilised to forecast the transmembrane region (https://services.healthtech.dtu.dk/services/TMHMM-2.0/), subsequently, the sequence was categorised into inside, out-

side, and transmembrane sections. The folding prediction was made via Protein Secondary Structure Predictor (PSIPRED) version 4.0. (http://bioinf.cs.ucl.ac.uk/psipred/).

Immunoinformatic analysis

Linear B-cell epitope prediction. The Lasota and/or phylogenetically significant strains from Indonesia were analysed to forecast the B-cell linear epitopes in the Immune Epitope Database (IEDB) at http://tools.iedb.org/bcell/ using the parameters of Kolaskar-Tongaonkar antigenicity, Emini surface accessibility, Bepipred 1.0, and Bepipred 2.0 using the given thresholds.

Linear T-cell epitope prediction. The prediction of both T-cytotoxic and T-helper linear epitopes is also performed using methods from IEDB. predictions for T cell epitopes in humans across various alleles of major histocompatibility complex class I (MHCI) and class II (MHCII) were made. The IEDB MHC I prediction tool, available at http://tools.iedb.org/mhci was used to predict MHC-I binding epitopes. The binding affinity of peptides to MHCI molecules was measured using the artificial neural networks (ANN) method. Before making predictions, the peptide lengths were defined as 8 to 10-mers (Wieczorek *et al.,* 2017). The IC50 values were established as less than or equal to 300 nM for the peptide's binding to MHC-I molecules. The IEDB MHCII prediction tool was utilised for the analysis of MHC class II molecules, accessible at http://tools.iedb.org/mhcii/. Human MHC class II alleles, specifically HLADR, HLADP, and HLADQ, were employed for the prediction of MHCII binding. The NN-align approach with IC50 values of 1000 nM or less was employed, while the peptide lengths were defined as 13 to 25-mers (Wieczorek *et al.,* 2017).

Antigenicity, allergenicity and toxicity of predicted epitopes. The linear epitopes that were predicted were subsequently evaluated using prediction parameters: assessing the antigenicity with Vaxijen 2.0. (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxyJen.html), allergenicity with AllerTop (https://www.ddg-pharmface.net/AllerTOP/), and toxicity with ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/). Only the epitopes that are antigenic, non-allergenic, and non-toxic will be utilised for further investigation (Martinelli, 2022).

Homology modelling and conformational B-cell epitop prediction. We also submitted previous sequences to the Swiss Model webserver (https://swissmodel.expasy.org/) to generate a quaternary 3-dimensional (3D) structure using the automated homology modeling method. The PyMol version 2.5.8 program enhanced the structure from the previous homology modeling output by eliminating non-protein components such as ligands. the Ramachandran plot of the modified setup was validated by analysing it using PROCHECK on the SAVES web server (https://saves.mbi.ucla.edu/). The IEDB webserver (http://tools.iedb.org/ellipro/) utilised ElliPro to predict conformational epitopes based on revised and validated structures. Once again the PyMol version 2.5.8 software was used to visually represent the confirmed structures and map the previously predicted B-cell epitopes.

Epitope conservancy calculation. The verified epitopes were calculated for epitope conservancy (http://tools.iedb.org/conservancy/) among Lasota and Indonesian strains, with 100% of the identity threshold.

Analysis of results

The data obtained in this study were examined using descriptive methods. The phylogenetic tree construction datawas used to determine the phylogenetic significance of the cluster and/or subclusters. The identity between the reference strain and Indonesia was calculated on the basis of genetic data. Amino acid polymorphism data were utilised to detect changes in amino acids at the antigenic sites (Iorio *et al.,* 1991) and previously predicted epitopes. The epitope conservancy was used to calculate the level of conservancy between the Lasota and Indonesia strains, then further classified these findings into conserved and non-conserved epitopes. More precisely, as previously discussed, three-dimensional structures also represent B-cell epitopes that are conserved and/or have phylogenetic relevance.

Fig. 1. The phylogenetic tree construction (neighbour-joining, 1000 bootstrap) of the amino acid sequence HN no. 1-555, results in two main clusters: GI-GIV originated and GV-VII originated. Isolates with a black round label are ND Indonesian isolates; isolates with a box label are Lasota reference isolates; isolates with dotted-line sub-cluster branches are ND isolates derived from wild birds.

RESULTS

The study of 555 HN amino acid sequences using phylogenetic analysis showed that the tree was split into two main clusters (Fig. 1), polymorphisms of important amino acids predicted to be immunogenic (Fig. 2), and the identity of the Lasota strain, which was 86.1–99.8% against Indonesian strains (Table 1). The phylogenetic tree (neighbour-joining, bootstrap 1000) divided the HN sequences of the tested isolates into two main groups: GV-GVII originated and GI-GIV originated (Fig. 1). Pesawaran isolate (MZ488470.1) is not in the same sub-cluster as Ulster isolate, both of which were characterised as GI based on F protein in previous research (Table 2). The conformations of GVII.2 isolates originated consistently in a GV-GVII cluster. Uniquely, the isolates from wild birds (peacocks and eagles) form a sub-cluster conformation (dotted-line branch, Fig. 1). Another finding, the BRS20 (MZ488472.1) (GII originated) isolate was identified most closely to Lasota, with a pairwise distance value of only 0.2% (1 of 555 different amino acids) and was in the same sub-cluster. The study of secondary structures helped to show how possible mutations might have affected. The mutated amino acid is $_{277}$F→L, which is only found in BRS20 isolate. These amino acids are thought to be outside the membrane (46–555) (Fig. 3A), along with antigenic sites and conserved epitopes, based on the transmembrane region prediction in this study. Regrettably, it did not fit into these categories, neither antigenic si-

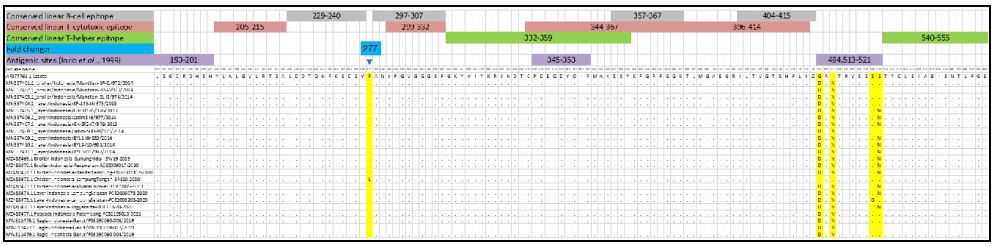

Fig. 2. Amino acid polymorphism of HN sequences for both the ND Lasota reference and Indonesia. The amino acids shown are the only ones that play a role in immunogenicity, based on the results of this study and previous research. The highlighted amino acids indicate mutations.

Table 1. The calculation result of pairwise distance and identity sequence of amino acid HN from the Lasota reference strain against ND isolates from Indonesia, both from commercial chickens and wild birds

Indonesian strain	Lasota					
	Distance			Identity		
GI originated	0.2%			99.8%		
GII originated	7.1%			92.9%		
GVII originated						
Overall	12.7%	–	13.9%	86.1%	–	87.3%
Commercial chicken	12.7%	–	13.9%	86.1%	–	87.3%
Wild bird	13.1%	–	13.9%	86.1%	–	86.9%

Table 2. List of HN sequences of ND isolates analysed in this study

Genbank Acc. Number	Isolate name	Previous genotype based on F protein	
AF077761.1	Lasota	GII	de Leeuw & Peeters, 1999
MN557401.1	broiler/Indonesia/Muntilan-1P-I1/972/2014	GVII.2	Goraichuk *et al.*, 2020
MN557402.1	broiler/Indonesia/Muntilan-2P-I2/973/2014	GVII.2	Goraichuk *et al.*, 2020
MN557403.1	broiler/Indonesia/Muntilan-2L-I3/974/2014	GVII.2	Goraichuk *et al.*, 2020
MN557404.1	layer/Indonesia/KP-145-I4/975/2013	GVII.2	Goraichuk *et al.*, 2020
MN557405.1	layer/Indonesia/GK-SR1-I5/976/2013	GVII.2	Goraichuk *et al.*, 2020
MN557406.1	layer/Indonesia/Jatim3-I6/977/2014	GVII.2	Goraichuk *et al.*, 2020
MN557407.1	layer/Indonesia/GK-SR2-I7/978/2013	GVII.2	Goraichuk *et al.*, 2020
MN557408.1	layer/Indonesia/Jatim-SDD-I8/979/2014	GVII.2	Goraichuk *et al.*, 2020
MN557409.1	layer/Indonesia/BYL1-I9/980/2014	GVII.2	Goraichuk *et al.*, 2020
MN557410.1	layer/Indonesia/BYL2-I10/981/2014	GVII.2	Goraichuk *et al.*, 2020
MN557411.1	layer/Indonesia/BYL3-I11/982/2014	GVII.2	Goraichuk *et al.*, 2020
MZ488469.1	Broiler-Indonesia-GunungKidul-ISW19-2019	GI	Angeliya *et al.*, 2022b
MZ488470.1	Broiler-Indonesia-Pesawaran-A032009017-2020	GVII.2	Angeliya *et al.*, 2022b
MZ488471.1	Chicken-Indonesia-BandarLampung-P032001007-2020	GVII.2	Angeliya *et al.*, 2022b
MZ488472.1	Chicken-Indonesia-LampungTengah-BRS20-2020	GII	Angeliya *et al.*, 2022b
MZ488473.1	Chicken-Indonesia-MuaraEnim-A031909005-2019	GVII.2	Angeliya *et al.*, 2022b
MZ488474.1	Layer-Indonesia-LampungSelatan-P032006078-2020	GVII.2	Angeliya *et al.*, 2022b
MZ488475.1	Layer-Indonesia-LampungSelatan-P032006203-2020	GVII.2	Angeliya *et al.*, 2022b
MZ488477.1	Peacock-Indonesia-Palembang-P032105010-2021	GVII.2	Angeliya *et al.*, 2023
MZ488476.1	Layer-Indonesia-Yogyakarta-DOCHTN20-2020	GVII.2	Angeliya *et al.*, 2022b
MW811479.1	Eagle-Indonesia-Garut/P08190060-004/2019	GVII.2	Angeliya *et al.*, 2022a
MW811477.1	Eagle-Indonesia-Garut/P08190018-013/2019	GVII.2	Angeliya *et al.*, 2022a
MW811475.1	Eagle-Indonesia-Garut/P08190060-005/2019	GVII.2	Angeliya *et al.*, 2022a

Fig. 3. Results of secondary structure analysis: a) THMM 2.0 predicts transmembranes from Lasota and BRS20 strains: inside membrane (1–22), transmembrane helix (23–45), and outside membrane (46–555); b) A random coil (shown by a gray label), a helix (shown by a pink label), and a strand (shown by a yellow label) are used to predict how proteins will fold using PSIPRED from Lasota strains and BRS20. Red boxed amino acids are a "fold changer", with mutation $_{277}$F→L from Lasota to Indonesian BRS20. Blue boxed letters are affected amino acids according to the "fold changer".

tes nor conserved epitopes (Fig. 2). We referred to the $_{277}$L residue in BRS20's HN as a "fold changer" because it altered the protein fold without altering its immunogenicity (Fig. 2 and Fig. 3B). Other mutations were also detected in antigenic sites: $_{494}$G→D and $_{514}$I→V (except BRS20), $_{520}$S→G (P032006203), and $_{521}$S→N (some isolates) (Fig. 2).

A total of the 449 epitopes (already screened to be antigenic, non-allergenic, and non-toxic) predicted in Lasota's HN, but only 40 epitopes (8.9%) of them were conserved in Indonesian isolates (n=23) (Fig. 4). All conserved epitopes are on the outer membrane based on the THMM 2.0 prediction (Fig. 2), while there is only one non-conserved epitope 11ENDEREAKNT20 predicted to be located on the inner membrane. The epitopes include epitopes that are predicted to function on humoral immune responses (linear and conformational B-cells) (Table 3, Fig. 5) and cellular immune responses (Tables 4 and 5), with the ratio of B-cell epitopes to T-cell epitopes being 1:33.53. In more detail, 4 out of 9 (44.4%) linear B-cells (Table 3, Fig. 5A), 0 out of 6 (0%) conformational B-cells (Fig. 5A), 30 out of 100 (30%) linear T-cytotoxic cells (Table 4), and 6 out of 366 (1.64%) linear T-helper cell epitopes (Table 5) were found to be conserved. In addition, the unique epitope $_{164}$PAPTTGTGCTR$_{174}$ was also found in Wildbird's GVII-originated NDV, which may have mutated from the Lasota NDV (Fig. 5B).

DISCUSSION

This study found that the molecular immunogenicity of the HN protein in the Indonesian NDV strain exhibits significant differences compared to the vaccine strain. Phylogenetic analysis showed that there were two main clusters (Fig. 1), reflecting a significant genetic diversity among Indonesian isolates and Lasota

Table 3. List of linear B-cell epitopes predicted from the IEDB server. There are 4 out of 9 conserved linear B-cell epitopes in Lasota, compared to 23 Indonesian strains

Method	No.	Antigenic, non-toxic, non-allergen B-cell epitopes	Conservancy	THMM 2.0*
Bepipred 2.0	1	140LIVDDASDVTSFYPSAFQEHLNFIPAPTTGS170	<100%	outside
	2	281YHEKDLDVTTL291	<100%	outside
Bepipred	3	229LDDTQNRKSCSV240	100%	outside
	4	297ANYPGVGGGSF307	100%	outside
	5	357SSYKPGRFGGK367	100%	outside
Emini surface accessibility	6	11ENDEREAKNT20	<100%	inside
Kolaskar-Tongaonkar	7	201HSYQYLALGVLRT213	<100%	outside
	8	371QAILSIKVSTS381	<100%	outside
	9	404ILTVGTSHFLYQ415	100%	outside

*Based on transmembrane prediction result with THMM 2.0.

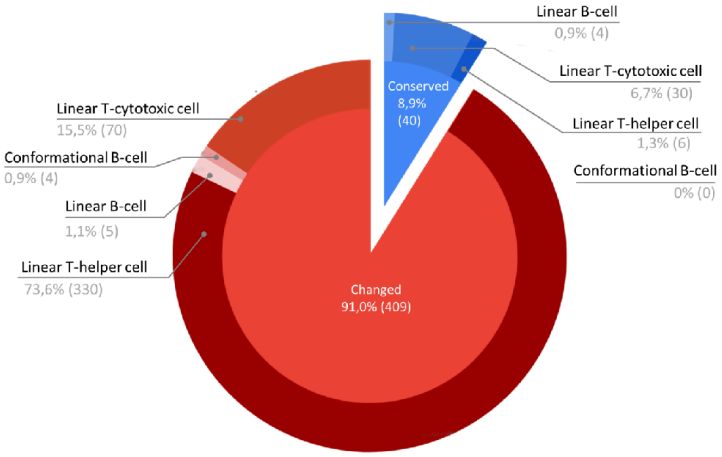

Fig. 4. Conservancy percentage of HN protein epitopes in the Lasota strain, both conserved and changed, compared to 23 isolates originating from Indonesia. The red area on the pie chart represents the percentage of conserved epitopes, and the blue area – the percentage of changed epitopes.

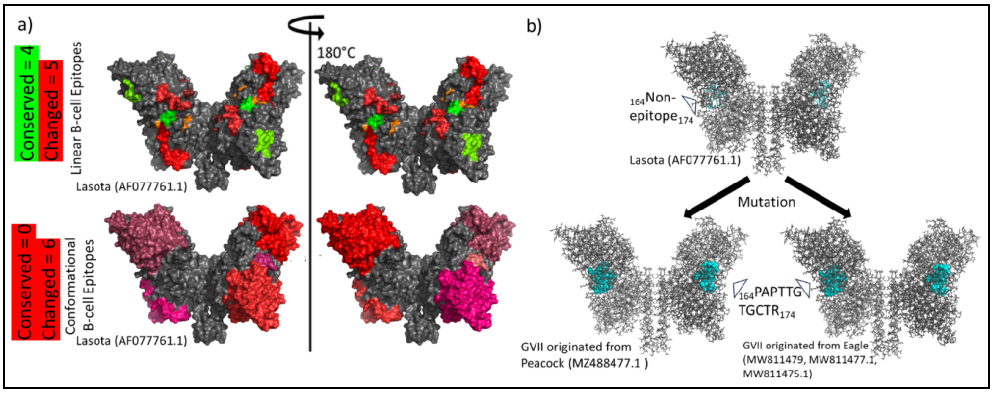

Fig. 5. The predicted 3D protein structure with epitope mapping: a) linear B-cell epitopes (upper side) and conformational B-cell epitopes (lower side). b) An example of mutation that changed a non-epitope site in Lasota into a B-cell epitope in the HN NDV that was found in wild birds in Indonesia.

strains. The identity between Lasota and the Indonesia strain ranged from 86.1% to 99.8%, indicating genetic variation that could affect the immune response. Some mutations in the HN protein, such as residue 277, can affect protein folding without reducing immunogenicity (Fig. 3). This variability may explain why the Lasota vaccine may have a lower level of protection against other Indonesian strain. Previous research has also shown that mutations at antigenic sites can alter the immunogenic profile of viruses (Chang *et al.,* 2023; Sheng *et al.,* 2024). Fig. 2 suggests that antigenic sites (including mutations in 494, 514, 520, and 521 residues) may experience hypervariability due to their exclusion from the conserved epitope prediction. This indicates those sites are

Table 4. A list of linear T-cytotoxic cell epitopes predicted by the IEDB server. There are 30 out of 100 conserved linear T-cytotoxic cell epitopes in Lasota were found to be conserved to Indonesian strains. Only conserved epitopes were displayed

No.	Start	Peptide	End	Length	MHC-I allele	ann IC50
1	205	YLALGVLRT	213	9	HLA-A*02:01	119.39
					HLA-A*02:03	24.08
2	205	YLALGVLRTS	214	10	HLA-A*02:03	85.21
3	206	LALGVLRTSA	215	10	HLA-A*02:06	239.07
4	299	YPGVGGGSF	307	9	HLA-B*07:02	16.51
					HLA-B*35:01	37.02
5	344	CPDEQDYQI	352	9	HLA-B*53:01	23.81
6	350	YQIRMAKSSY	359	10	HLA-A*30:02	167.28
					HLA-B*15:01	6.86
7	351	QIRMAKSSY	359	9	HLA-A*30:02	272.2
					HLA-B*15:01	95.94
8	353	RMAKSSYKPG	362	10	HLA-B*15:01	181.76
9	353	RMAKSSYK	360	8	HLA-A*03:01	60.91
					HLA-A*31:01	69.56
10	354	MAKSSYKPGR	363	10	HLA-A*31:01	32.97
					HLA-A*68:01	45.74
11	354	MAKSSYKPG	362	9	HLA-A*30:01	80.33
12	358	SYKPGRFGGK	367	10	HLA-A*31:01	170.5
					HLA-A*30:01	288.43
13	396	TLMGAEGRI	404	9	HLA-A*02:03	100.02
14	405	LTVGTSHFLY	414	10	HLA-B*15:01	134.62
					HLA-A*68:01	219.36
					HLA-B*58:01	227.73
					HLA-A*30:02	40.74
					HLA-A*01:01	50.26
15	405	LTVGTSHFL	413	9	HLA-B*58:01	180.56
					HLA-A*68:02	19.5
					HLA-A*02:06	75.2
16	406	TVGTSHFLY	414	9	HLA-A*11:01	117.88
					HLA-A*30:02	190.05
17	413	LYQRGSSYFS	422	10	HLA-A*24:02	201.82
					HLA-A*23:01	244.68
18	414	YQRGSSYF	421	8	HLA-B*15:01	101.76
19	418	SSYFSPALLY	427	10	HLA-A*11:01	18.03
					HLA-A*03:01	25.15
					HLA-A*30:02	28.07
					HLA-A*01:01	70.02
20	418	SSYFSPALL	426	9	HLA-A*68:02	89.02
21	419	SYFSPALLY	427	9	HLA-A*30:02	48.73
22	467	TGVYTDPYPL	476	10	HLA-A*68:02	188.98
23	468	GVYTDPYPL	476	9	HLA-A*02:06	211.17
					HLA-A*02:03	87.13
					HLA-A*02:01	94.15
24	469	VYTDPYPLIF	478	10	HLA-A*23:01	14.15
					HLA-A*24:02	27.59
					HLA-A*01:01	31.39
25	469	VYTDPYPLI	477	9	HLA-A*24:02	51.35
					HLA-A*23:01	63.98
26	470	YTDPYPLIF	478	9	HLA-B*58:01	43.4
					HLA-A*01:01	6.07
27	526	YTTSTCFKVV	535	10	HLA-A*68:02	126.49
					HLA-A*02:06	180.01
28	527	TTSTCFKVVK	536	10	HLA-A*03:01	167.81
					HLA-A*68:01	21.48
					HLA-A*11:01	25.91
29	527	TTSTCFKVV	535	9	HLA-A*68:02	37.99
30	528	TSTCFKVVK	536	9	HLA-A*68:01	24.51
					HLA-A*31:01	258.84
					HLA-A*11:01	60.19

Table 5. A list of linear T-helper cell epitopes predicted by the IEDB server. There are 6 out of 366 linear T-helper cell epitopes in Lasota were found to be conserved to Indonesian strains. Only conserved epitopes were displayed

Core_peptide	Allele	Epitope No.	Start	Peptide	End	Length	IC50
KYVIYKRYN	HLA-DQA1*05:01/DQB1*04:02	1	332	GKYVIYKRYNDTCPDEQDYQIRMA	355	24	942.2
DYQIRMAKS	HLA-DQA1*01:02/DQB1*06:02	2	335	VIYKRYNDTCPDEQDYQIRMAKS	357	23	396.3
	HLA-DRB1*10:01	2	335	VIYKRYNDTCPDEQDYQIRMAKS	357	23	825
	HLA-DQA1*01:02/DQB1*06:02	2	335	VIYKRYNDTCPDEQDYQIRMAKSS	358	24	123.7
	HLA-DRB1*10:01	2	335	VIYKRYNDTCPDEQDYQIRMAKSS	358	24	541.7
	HLA-DRB1*16:02	2	335	VIYKRYNDTCPDEQDYQIRMAKSS	358	24	634.8
	HLA-DQA1*01:02/DQB1*06:02	3	335	VIYKRYNDTCPDEQDYQIRMAKSSY	359	25	88.4
	HLA-DRB1*10:01	3	335	VIYKRYNDTCPDEQDYQIRMAKSSY	359	25	154.6
KRYNDTCPD	HLA-DPA1*01:03/DPB1*03:01	3	335	VIYKRYNDTCPDEQDYQIRMAKSSY	359	25	888
EISNTLFGE	HLA-DPA1*01:03/DPB1*02:01	4	540	TYCLSIAEISNTLFGE	555	16	770.2
	HLA-DPA1*02:01/DPB1*01:01	4	540	TYCLSIAEISNTLFGE	555	16	186
LSIAEISNT	HLA-DQA1*05:01/DQB1*03:01	5	540	TYCLSIAEISNTLF	553	14	487.8
	HLA-DRB3*03:01	5	540	TYCLSIAEISNTLF	553	14	102.8
	HLA-DQA1*01:02/DQB1*06:02	4	540	TYCLSIAEISNTLFGE	555	16	859.4
	HLA-DQA1*05:01/DQB1*03:01	4	540	TYCLSIAEISNTLFGE	555	16	325.4
	HLA-DRB3*03:01	4	540	TYCLSIAEISNTLFGE	555	16	77.8
SIAEISNTL	HLA-DQA1*01:03/DQB1*06:03	6	540	TYCLSIAEISNTLF	553	14	577
	HLA-DRB1*04:04	6	540	TYCLSIAEISNTLF	553	14	53.1
	HLA-DRB1*16:02	6	540	TYCLSIAEISNTLF	553	14	609.4
	HLA-DQA1*01:03/DQB1*06:03	4	540	TYCLSIAEISNTLFGE	555	16	476.3
	HLA-DRB1*04:04	4	540	TYCLSIAEISNTLFGE	555	16	102.4
	HLA-DRB1*16:02	4	540	TYCLSIAEISNTLFGE	555	16	324.9

more susceptible to mutations, which could be the virus' mechanism to evade recognition by the host immune system (Chang *et al.,* 2023).

These differences in immunogenicity may contribute to the insufficient protection observed in vaccinated poultry, leading to ND recurrence in Indonesia. The recurrence of ND outbreaks in vaccinated poultry suggests that the current vaccines may not fully cover the antigenic diversity present in the circulating field strains, as evidenced by significant differences in the haemagglutinin-neuraminidase (HN) protein between the vaccine strain and the Indonesian NDV strains (Rabiei *et al.,* 2020). Serological analyses have shown that neutralising antibody titres in vaccinated birds are significantly lower against the circulating strain compared to the vaccine strain, indicating that the immune response generated by the vaccine may be insufficient to prevent infection (Seung *et al.,* 2022). Vaccines may not protect as well when there are differences in the HN protein antigens, especially in the epitope regions (Tables 3, 4, 5; Fig. 5), in line with study that found about distinct immunogenicity (Chang *et al.,* 2023). There are still virulent NDV sub-genotypes, like VII.2, in vaccinated chicken populations. This suggests that the current vaccine strain doesn't make enough of an immune response to prevent the new variants (Goraichuk *et al.,* 2020; Pandarangga *et al.,* 2020). The genetic analysis of NDV strains found in recent outbreaks in Indonesia showed major changes in the HN protein (Fig. 2, 4; Table 2) that could help the virus avoid the immune system, may explain why ND persists despite efforts to prevent it (Doan *et al.,* 2020; Rabiei *et al.,* 2020).

The variation in antigenic sites and predicted epitopes suggests that the vaccine strain's immune response may not be as effective in neutralising the Indonesian strain. Fig. 4 can give an idea that there are many epitopes of the Lasota vaccine, both humoral and cellular, that have the potential to be unrecognised by the immune response of poultry, even those which are well-vaccinated. Cellular immune responses, particularly those involving T cells, are often difficult to calculate accurately (Ojha & Prajapati, 2021). Mutations in antigenic sites (Table 1) in HN proteins found in Indonesian strains may not be fully recognised by the immune response to the epitopes presented from the vaccine antigen processing mechanism, both through MHC-I and MHC-II (Wieczorek *et al.,* 2017), whereas it may be only epitopes conserved on vaccines (Table 3, 4, 5; Fig. 5A) that can still be recognised. As well as the B-cell epitope to the humoral response that has changed a lot (Table 3; Fig. 5), this is in line with the postulate proposed by previous research that it is necessary to use antigens that match the circulating virus in Indonesia, both for vaccination purposes and for monitoring with the serological test of hemagglutination-inhibition (HI) (Pandarangga *et al.,* 2020). Ultimately, NDV isolated from wild birds, eagles and peacocks (Angeliya *et al.,* 2022a; 2023), showed significant mutations in the HN epitope (Fig. 5B), which allowed the virus to evade recognition by the immune system of vaccinated poultry (Babaeimarzangou *et al.,* 2023; Kalonda *et al.,* 2024). Other mutations in HN proteins found in NDV isolates from wild birds, such as antigenic site substitution (Fig. 2), indicate changes in antigenic sites that could potentially reduce the effectiveness of current NDV vaccines. These findings are consistent with results suggesting that epitopes (Fig. 5B) in wild birds can evade

recognition by antibodies produced by vaccines. These genetic differences emphasise the need for a vaccine update that includes epitopes of NDV-originating wild birds to improve protection against reinfection (Putri *et al.,* 2021; Babaeimarzangou *et al.,* 2023).

These findings suggest that updating or supplementing the current ND vaccines to include epitopes more representative of the Indonesian NDV strain may be necessary. Although Indonesia has made efforts to develop ND vaccines that are suitable for local strains such as Suminta, B1, Lasota, Komarov, and genotype-based (GI, GII, and GVII), it turns out that there are still significant challenges in ensuring the effectiveness of vaccination in the field, especially due to the ongoing evolution of the virus (Dharmayanti *et al.,* 2024). In Indonesia, work on developing a vaccine for ND is mostly focused on studying how the virus changes at the molecular level on the F protein (Ansori *et al.,* 2021; Angeliya *et al.,* 2023; Dharmayanti *et al.,* 2024) in order to provide clinical protection and lower viral shedding (Cahyani *et al.,* 2020; Dharmayanti *et al.,* 2024). The immune response to the epitope on the HN protein is not given as much attention. The *in-silico* approach to vaccine development in Indonesia so far has also focused on F protein only (Putri *et al.,* 2021), while the results of this study can be the basis for the importance of studying the molecular immunogenicity of HN protein.

The results of this study are not only relevant for Indonesia, but also have significant global implications in the control of NDV. The genetic and antigenic variations found in the HN protein of NDV reflect the global challenges faced in the development of effective vaccines. In different countries, differences in genotype and antigenicity of NDV often lead to a decrease in the efficacy of existing vaccines (Choi *et al*, 2013; Mase, 2022). Therefore, the immunoinformatics-based approach used in this study can be an important framework for updating vaccines, not only in Indonesia but also in other regions with similar challenges. This increased understanding of molecular immunogenicity could also accelerate the development of a broader-covered vaccine, providing more effective protection against NDV strains that continue to evolve globally.

Future research should focus on developing more targeted vaccines, considering the molecular immunogenicity differences highlighted in this study, to improve ND control in endemic regions like Indonesia. As we already said, when Indonesian scientists had made ND vaccines, they did not thought much about how HN proteins affect humoral and cellular immunity (Table 3, 4, 5; Fig. 5A). In addition to the molecular considerations of circulating viruses in the development of conventional ND vaccines in Indonesia (Dharmayanti *et al.,* 2024), various studies of non-conventional vaccines that consider the immune response to have a positive effect. Vaccines that can boost both humoral (which neutralises virus) and cellular (which stops virus shedding) immune responses work better (Bello *et al.,* 2018; Hu *et al.,* 2022). In situations where there is high variability from mutated viruses, vaccines that stimulate cellular immune responses may reduce reliance on antibody responses alone, which may not always be adequate to provide long-term protection (Bello *et al.,* 2018; Rajab *et al.,* 2024). For instance, recombinant vaccines, specifically designed to target specific epitopes recognised by T cells, can offer superior protection in comparison to

conventional vaccines, allows the vaccine to better suit the virus's molecular variation, thereby increasing its overall efficacy (Rajab *et al.,* 2024). Those previous studies demonstrate that vaccines that are more responsive to the evolution of viral molecular immunogenicity can improve control and effectiveness in preventing virus spread.

CONCLUSION

In conclusion, there were significant differences in the molecular immunogenicity between the Lasota strain and the circulating Indonesian NDV. This study also predicts that the ND Lasota vaccine's low level of protection against local Indonesian strains may contribute to the recurrence of ND cases, even in vaccinated poultry. Further, a HN molecular immunogenicity-based vaccine with its *in vivo* test shall be done for the more comprehensive prevention of ND in Indonesia.

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