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Research Reports

Characterization of Amino Acid Mutations of Newcastle Disease Virus (NDV) In Swan Geese *(Anser cygnoides)* In East Java, Indonesia

Karakterisasi Mutasi Asam Amino Virus Newcastle Disease pada Angsa (Anser cygnoides) di Jawa Timur, Indonesia

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ABSTRACT

Background: Newcastle disease (ND) is a highly contagious viral disease affecting the poultry industry. Newcastle Disease Virus (NDV) is classified into three strains based on their relative virulence, namely velogenic or highly virulent, mesogenic or moderately virulent, and lentogenic or lowly virulent. The clinical manifestations of the disease vary depending on many factors, such as host susceptibility and the virulence of the NDV strain. **Purpose:** This study aims to analyze the amino acid mutations of the NDV in unvaccinated swan goose *(Anser cygnoides)* from various locations in Java. **Methods:** Samples were collected through cloacal swabs and isolated by inoculation in Specific Pathogen-Free (SPF) embryonated eggs that were nine days old. Hemagglutination and hemagglutination inhibition tests were conducted to confirm that the isolated virus was NDV. The isolated virus was processed using reverse transcription-polymerase chain reaction (RT-PCR) with primers that amplified partial sequences of the fusion (F) gene, which was analyzed to determine the pathotype. **Results:** The results indicated the presence of mutations in several regions. The amino acid changes occurred in 17 variable sites (7.2%) between RefSeq/JF950510 and ND/SW1/2018, 12 variable sites (5.1%) between RefSeq/JF950510 and ND/SW2/2018, 13 variable sites (5.5%) between RefSeq/JF950510 and ND/SW3/2018, and 19 variable sites (8.1%) between RefSeq/JF950510 and ND/SW4/2018. The amino acid sequences of the cleavage site of the fusion (F) protein revealed that all isolates had low virulence. **Conclusion:** The results indicated that mutations in the region outside the cleavage site not were incapable of altering the virulence of the virus.

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ABSTRAK

Latar Belakang: Newcastle disease (ND) adalah penyakit virus yang sangat menular yang mempengaruhi industri perunggasan. Virus Newcastle disease (NDV) diklasifikasikan menjadi tiga strain berdasarkan virulensi relatifnya, yaitu velogenik atau sangat virulent, mesogenic atau cukup virulent, dan lentogenic atau virulen rendah. Manifestasi klinis penyakit bervariasi tergantung pada banyak faktor, seperti kerentanan inang dan virulensi strain NDV. **Tujuan:** Penelitian ini bertujuan untuk menganalisis mutasi asam amino NDV pada angsa *(Anser cygnoides)* yang tidak divaksinasi dari berbagai lokasi di Jawa. **Metode:** Sampel dikumpulkan melalui usap kloaka dan diisolasi dengan inokulasi pada telur berembrio Bebas Patogen Spesifik (SPF) yang berumur sembilan hari. Tes penghambatan hemaglutinasi dan hemaglutinasi dilakukan untuk mengkonfirmasi bahwa virus yang diisolasi adalah NDV. Virus yang diisolasi diproses menggunakan *reverse transcription-polymerase chain reaction* (RT-PCR) dengan primer yang memperkuat urutan parsial gen fusi (F), yang dianalisis untuk menentukan patotipe. **Hasil:** Hasilnya menunjukkan adanya mutasi di beberapa daerah. Perubahan asam amino terjadi di 17 situs variabel (7,2%) antara RefSeq / JF950510 dan ND / SW1 / 2018, 12 situs variabel (5,1%) antara RefSeq / JF950510 dan ND / SW2 / 2018, 13 situs variabel (5,5%) antara RefSeq / JF950510 dan ND / SW3 / 2018, dan 19 situs variabel (8,1%) antara RefSeq / JF950510 dan ND / SW4 / 2018. Sekuens asam amino dari situs pembelahan protein fusi (F) mengungkapkan bahwa semua isolat memiliki virulensi rendah. **Kesimpulan:** Hasilnya menunjukkan bahwa mutasi di daerah di luar lokasi pembelahan tidak mampu mengubah virulensi virus.

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Kata kunci: *Anser cygnoides;* Asam Amino, Mutasi; Newcastle Disease Virus; Virulensi

INTRODUCTION

Newcastle disease virus (NDV) belongs to the Paramyxoviridae family and is classified within the Avulavirinae subfamily, the Orthoavulavirus genus, and the Avian orthoavulavirus species (Amarasinghe *et al.,* 2018, Kuhn *et al.,* 2019). NDV has a broad host range, capable of infecting over 240 species of birds (Umali *et al.,* 2014). An enveloped, negative-sense, single-stranded RNA virus, it has approximately 15,000 nucleotides and causes explosive outbreaks of severe diseases in poultry species (Bagoyav lenskiy *et al.,* 2005). The initial outbreaks of ND in Java, Indonesia, and Newcastle-upon-Tyne, England, were reported during the mid-1920s (Doyle 1927, Kraneveld, 1926). According to data from the World Organization for Animal Health (WOAH), NDV is a notifiable disease in the poultry industry (WOAH, 2018).

The NDV belongs to a single serotype and comprises two classes. The genomes of class I and class II viruses consist of 15,198 nucleotides (nt) and 15,186 or 15,192 nt, respectively (Czegledi *et al.,* 2006). Six open reading frames (ORFs) encode the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L). The P, L, and M polymerase complexes influence the virulence and replication of viruses in chickens (Ji *et al.,* 2018). RNA editing during P gene transcription generates at least one additional, non-structural protein (V) and potentially a second one (W). The NDV has two major glycoproteins that are involved during NDV infection, namely the fusion (F) and hemagglutinin-neuraminidase (HN) proteins (Samal, 2012). The F protein has a length of length 1,729 base pairs (bp) and is typically analyzed for genotyping NDV (Miller *et al.,* 2015). The HN protein is responsible for inducing antibody (Absalon *et al.,* 2019).

The NDV is an RNA virus. Errors that occurr during its replication facilitate the mutation of the RNA virus, making it evolve quickly (Jenkins *et al.,* 2002). Changes in the virulence of NDV are caused by mutations in the amino acids at the cleavage site of the fusion protein. Therefore, it is necessary to analyze the amino acid mutations of the NDV in swan geese.

MATERIAL and METHOD

Sample Collection and Virus Isolation

A total of 71 samples were collected from healthy swan geese in East Java. The swab samples were transferred to 1.5 mL centrifuge tubes containing 1.0 mL of transport medium (50% glycerol in phosphate-buffered saline) and antibiotics (2000 IU/mL of penicillin and 2 mg/mL of streptomycin). All samples were isolated from the swab samples by standard virus isolation methods in embryonated chicken eggs (WOAH, 2012). Three embryonated chicken eggs that were nine to 11 days old were used for inoculation (Alexander and Senne, 2008). The inoculated eggs were candled every 24 hours to assess embryo vitality. On each day, eggs containing a dead were removed from the incubator and chilled at +4°C overnight. The allantoic fluids were harvested and tested by the hemagglutination (HA) test to determine their ability to hemagglutinate chicken red blood cells (RBCs).

HA and HI Test

The HA test was performed using a microtiter plate in accordance with the methodology outlined by the WOAH (2012). HA-positive samples were subjected to a hemagglutination inhibition (HI) test, which specifically identifies the agents responsible for hemagglutination.

Viral RNA Extraction

The RNAs of the viruses were extracted from allantoic fluids using the TRIzol LS Reagent (Invitrogen, Carlsbad, CA) following the provided instructions. A sample suspension was used for RNA extraction prior to storage at -80°C. Before PCR amplification, the RNA extraction products were quantified using the Qubit Assays.

Reverse Transcription-Polymerase Chain Reaction

Following the process of RNA extraction was the amplification of the partial F gene. Primers of specific oligonucleotides for RT-PCR amplification were sourced from PT Genetika Indonesia. The primers flanked the region in the cleavage site into the protein-coding gene of NDV. The sequences of the oligonucleotide primers are presented in **Table 1**. The conditions for the RT-PCR amplification of the complete F genes were as follows: the temperature was set at 95°C, with the lid on, followed by pre-denaturation at 94°C for 10 minutes, denaturation at 94°C for one minute, annealing at 57°C for one minute, and a final extension step at 72°C for 10 minutes. The PCR products were analyzed using 1.5% agarose gel electrophoresis.

Table 1. Sequences of oligonucleotide primers by RT-PCR for the amplification of the NDV F gene.

Primer	Sequence 5'-3'	Amplified Gene Size (bp)
Forward	GAC CGC TGA CCA CGA GGT TA	699
Reverse	AGT CGG AGG ATG TTG GCA GC	

Molecular Sequencing Analysis

The PCR products and the target band were purified and sequenced using an automatic ABI 3730XL DNA Analyzer (Applied Biosystem, Foster City, CA, USA).

Data Analysis

Molecular analysis is a method of combining and comparing nucleotide sequences that have been read by a sequencing machine. The nucleotide sequences were analyzed using Biological Sequence Alignment Editor (BioEdit) version 7.0.5.3.

RESULT

Virus Isolation and Identification

All samples were inoculated in allantoic fluids from Specific Pathogen-Free (SPF) embryonated chicken eggs that were nine to 10 days old for five days and were observed every 24 hours. Cloacal swabs were taken as NDV is known to be transmitted through the digestive and respiratory systems (WOAH 2012). Four samples showed positive results for HA,

namely ND/SW1/2018, ND/SW2/2018, ND/SW3/2018 and ND/SW4/2018. Samples with positive HA were confirmed by HI (haemagglutinin inhibition) as outlined in **Table 2.**

Table 2. HA and HI test results.

Samples	Identification Test	
	HA	HI
ND/SW1/2018	+	+
ND/SW2/2018	+	+
ND/SW3/2018	+	+
ND/SW4/2018	+	+
LaSota Positive Isolate	+	+

Mutational Analysis

The nucleotide sequences were analyzed into amino acids using BioEdit version 8.0. One amino acid was encoded by three nucleotides, resulting in a length of 699 nucleotides or equivalent to 233 amino acids. The translation of the amino acid sequence of each sample was continued through alignment using the ClustalW program integrated in BioEdit. The nucleotide and amino acid mutations (**Figures 1 and 2**) were analyzed to determine the effect of nucleotide mutations on amino acid sequences. **Table 3** shows the comparison of the changes in amino acids between RefSeq and the ND/SW1/2018, ND/SW2/2018, ND/SW3/2018, and ND/SW4/2018 samples.

The results indicated that amino acid mutations occurred in 17 variable sites (7.2%) between RefSeq/JF950510 and ND/SW1/2018, 12 variable sites (5.1%) between RefSeq/-JF950510 and ND/SW2/2018, 13 variable sites (5.5%) between RefSeq/JF950510 and ND/SW3/2018, and 19 variable sites (8.1%) between RefSeq/JF950510 and ND/SW4/2018. Variable sites are regions that can be mutated. This can be determined by performing a multiple alignment. The results of the multiple alignment using ClustalW integrated in BioEdit showed changes in several amino acids. In contrast, conserved sites are regions that do not change.

Evolutionary distance refers to the number of differences or mutations that occur between viruses. Zero evolutionary distance indicates no difference. Meanwhile, a value greater than zero indicates a difference or mutation. Table 4 shows that the biggest evolutionary distance was found in the ND/SW4/2018 sample. The distance between ND/SW4/2018 and RefSeq/JF950510 was 0.046, while the distance between ND/SW4/2018 and ND/SW1/2018 was 0.062. In addition, the distance between ND/SW4/2018 and ND/SW2/2018 was 0.037, while the distance between ND/SW4/2018 and ND/SW3/2018 was 0.037. This indicated that the ND/SW4/2018 sample had a big evolutionary distance from the vaccine strain (RefSeq/JF950510) and the other samples.

DISCUSSION

Newcastle disease poses a significant challenge for the poultry industry, with serious economic implications. The NDV from ducks in Indonesia carry various genotypes and patotypes that can potentially influence the rate of evolution of the NDV (Putri *et al.,* 2020). The NDV that grows in the allantoic fluid is tested for the presence of HA. Positive results inficate the presence of HA, causing chicken erythrocytes to agglutinate. Meanwhile, the HI test is based on the principle that the hemagglutinin present on the viral envelope can agglutinate chicken erythrocytes (red blood cells) and that this process can be inhibited by specific antibodies. HA from the NDV can bind specifically to sialic acid on the surface of sensitive cell receptors and facilitate the infection process. This delicate receptor is also present in chicken erythrocytes (Aris *et al.,* 2013).

The NDV in chickens can be divided into five pathotypes: asymptomatic enteric, lentogenic or respiratory, mesogenic, neurotropic velogenic, and viscerotropic velogenic (Alexander *et al.,* 1999). The lentogenic pathotype has a single basic amino acid motif at the cleavage sites of F 112G/EK/RG-G/E-R116, with L (leucine) at residue 117. Protease enzymes, such as trypsin, which are found in the digestive and respiratory tract have the potential to cleave this site (Choi *et al.,* 2010). In contrast, the mesogenic and velogenic pathotypes have multiple basic amino acid motifs (arginine or lysine) at the cleavage sites of 112R/K-R-Q/K/R-K/R-R116, with F (phenylalanine) at residue 117 (Meulemans *et al.,* 2002).

The amino acids present at the cleavage site indicated that the ND/SW1/2018, ND/SW2/2018, ND/SW3/2018, and ND/SW4/2018 samples as well as the LaSota positive isolates had a cleavage site structure similar to that of lentogenic pathotypes. The motif was a single basic amino acid 112G/EK/RGG/E-R116 with L (leucine) at residue 117. The region outside the cleavage site did not alter the virulence despite the presence of amino acid mutations (Putri *et al.,* 2019).

The smallest mutations are point mutations, in which only a single base pair is changed into another base pair. Another type of mutation is known as nonsynonymous mutations, which are changes in the amino acid sequence (Alam, 2013). The mutation ability of RNA viruses is faster than that of DNA viruses, due to the fact that they have a single single-stranded genome, in contrast to the double-stranded genome of DNA viruses. Furthermore, the mutation rate is negatively correlated with genome size. Viral mutation can be caused by the capacity of the virus to repair DNA mismatches and polymerase errors by proofreading and/or post-replication repairing (Sanjuan and Domingo-Calap, 2016). The polymerase enzyme of RNA viruses is characterized by low fidelity, making them easily mutated. In addition, each mutant within the quasispecies is genetically linked (Lauring and Andino, 2010).

CONCLUSION

It can be concluded that the RNA viruses were easily mutated and that some amino acid mutations in the region outside the cleavage site were incapable of altering the virulence of the virus.

Table 3. Mutations between amino acids, RefSeq, and samples.

No	Position	RefSeq/JF950510	ND/SW1/2018	ND/SW2/2018	ND/SW3/2018	ND/SW4/2018
1	9	Y	H	H	H	-
2	16	H	N	-	-	-
3	17	P	-	-	L	L
4	29	S	Q	N	N	N
5	30	L	F	-	-	-
6	31	L	-	I	-	-
7	32	D	R	-	-	-
8	33	C	-	G	G	G
9	34	A	M	R	A	-
10	40	E	D	E	D	D
11	195	N	G	G	G	G
12	203	A	-	-	-	I
13	205	W	-	-	-	C
14	206	L	-	-	-	I
15	208	G	-	-	-	I
16	209	L	F	-	-	V
17	210	Q	-	-	-	I
18	211	L	-	-	-	P
19	212	L	P	P	A	P
20	213	H	-	-	C	-
21	215	K	L	L	-	-
22	216	Q	E	E	K	K
23	217	R	E	-	Q	K
24	218	P	G	G	R	G
25	219	Q	H	-	-	-
26	221	K	L	-	-	-
27	223	K	M	M	T	M
28	224	P	-	-	-	L
29	225	N	-	-	-	R
Total			17	12	13	19

Table 4. Evolutionary distance between samples.

Samples	1	2	3	4
Refseq/JF950510				
ND/SW1/2018	0.041			
ND/SW2/2018	0.031	0.032		
ND/SW3/2018	0.032	0.045	0.022	
ND/SW4/2018	0.046	0.062	0.037	0.037

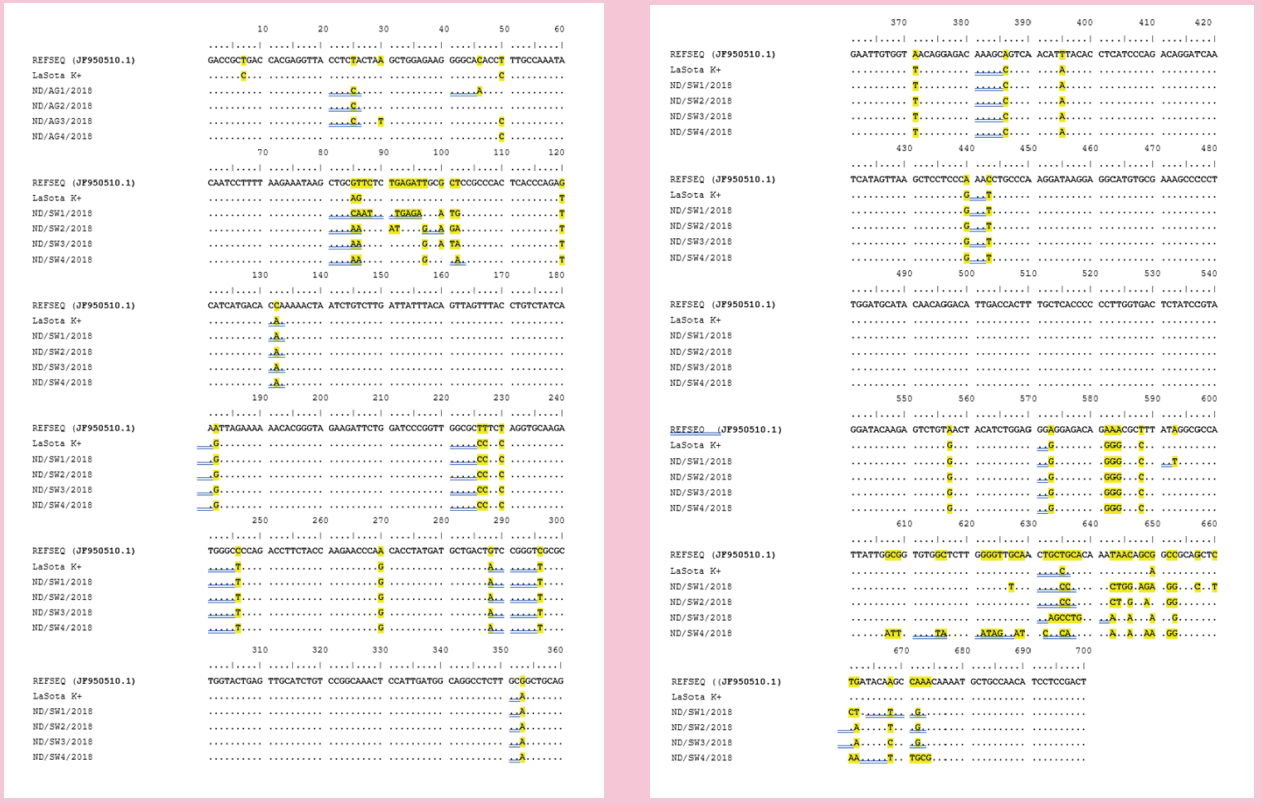

Figure 1. Nucleotide mutation of the gene encoding the NDV F protein

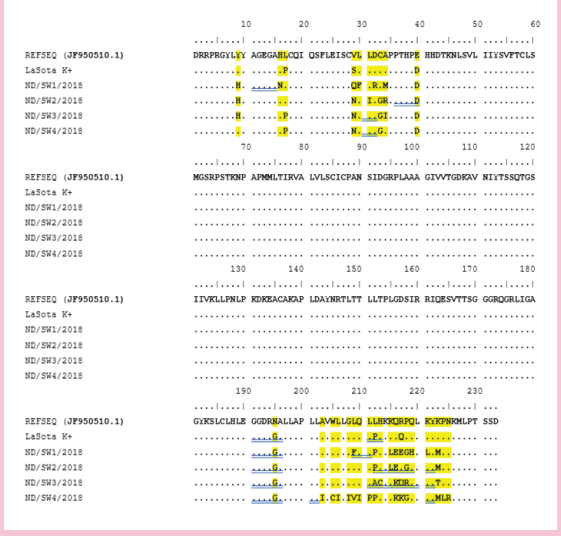

Figure 2. Amino acid mutation of the gene encoding the NDV

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CONFLICT of INTEREST

The author declares no conflict of interest.

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ETHICAL APPROVAL

The study did not require ethical approval. However, samples were collected in accordance with standard collection methods without causing any harm and stress to the animals.

AUTHORS' CONTRIBUTIONS

NP planned and carried out the study (collected samples and prepared the manuscript). IK analyzed the data and revised the manuscript. All authors approved the final version of manuscript.

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