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Nucleotide mutation analyses of isolated lentogenic newcastle disease virus in live bird market

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ABSTRACT

Newcastle Disease (ND) is a major viral disease in Indonesia. It is an RNA virus belongs to Paramyxovirinae. It is well known that RNA virus is easily to mutate. In some cases, this mutation could generate virulence alteration. It is noted that mutation of NDV which has avirulent amino acid sequence on the cleavage site, could mutate to be virulent Newcastle Disease Virus (NDV). It is needed to analyze the nucleotide and amino acid mutations and the effect of those to its virulence. The aim of this study was to analyze nucleotide and amino acid mutations of original isolated Lentogenic Newcastle Disease Virus (NDV). Samples were collected from cloacal swab of native chicken (Gallus gallus domesticus) suspected to be infected by Lentogenic NDV from live bird markets. They were inoculated into embryonated eggs, to isolate the virus. HA and HI assays were conducted to confirm that they were NDV. Positive samples were processed into serial passages in embryonated egg to observe their death time. Samples caused mortality of the embryonated eggs more than 90 hours post infection were suspected as Lentogenic NDV. They were processed to RT-PCR then sequenced. Lentogenic NDV confirmation was done by comparing amino acid at Fusion protein cleavage site of the samples to Lasota/JF950510. Nucleotide and amino acid mutations were analyzed. The result showed that some nucleotide mutations were capable to change sequences of amino acid but the virulence of the samples remained the same to the reference sequence.

Keywords: NDV; RNA virus; mutations; virulence

INTRODUCTION

Avian Paramyxovirus serotype 1 which known as Newcastle Disease Virus (NDV) is negative single stranded RNA virus belongs to Genus Avulavirus, family Paramyxovirinae [1]. The genome of NDV has length 15,186 bp encoding six major structural protein 3-N-P-M-F-

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HN-L-5 [2]. NDV is classified into three major strains based on clinical signs; Velogenic, Mesogenic, and Lentogenic [3]. Transcription of NDV is conducted in 3' to 5' direction [4]. Nucleoprotein (N) is firstly transcribed during replication [5]. Its length is 1,754 bp [2]. It protects genome of NDV and its positive sense from nucleases by the host cell [6]. Phospoprotein (P) has length 1,451 bp [2]. It takes part during transcription [6] and prevents formation of unexpected nucleocapsid during replication [7]. RNA editing during P protein transcription produces V and W proteins [4]. Both proteins are virulent factor for NDV pathogenesis [6]. Matrix protein (M) has length 1,241 bp [2]. It controls RNA synthesis, assembling and budding of NDV virion. M protein is conserved protein among Paramyxovirinae family [5]. Sequence of M protein encoding gene has 20% homology compared to other Paramyxoviruses M protein [8].

NDV has two major glycoproteins that involve during NDV infection; Fusion (F) and Haemagglutinin-neuraminidase (HN) proteins [9]. F protein is type I integral protein which has length 1,729 bp that vary among Newcastle Disease. It is usually analyzed for genotyping NDV [10]. Activation of F protein is not dependent low acidity in endosomal compartment. NDV enter the host cell by direct fusion [11]. HN protein is type II integral protein which has length 2,002 bp. It has several functions such as virion-receptor binding [12], elusion of progeny virus from its host cell receptor, and Fusion protein activation [13]. The last transcribed gene is Polymerase protein (L). It has length 2,204 bp [2]. L, N, and P proteins form Ribonucleo-Protein complex for RNA synthesis [6]. Each gene is separated by intergenic regions (IGs) vary in length (1-47 nt) [2]. Sequence of IGs are not same among NDV but it usually ended with adenosine [14]. NDV is RNA virus [2]. Error that occurred during its replication causes RNA virus easily to mutate. It makes RNA virus is evolved quickly [15]. The mutation of amino acid in cleavage site of Fusion Protein of Newcastle Disease Virus caused the virulence of the virus is changed. It was proofed by outbreak in Australia during 1998-2000 [16]. This rapid mutation also forms a viral quasispecies. Viral quasispecies is dynamic distribution of virus that have same lineage but not identic as the result of genetic variation process, competition, and selection [2]. This study aims to analyze mutation that occurred in Lentogenic NDV.

MATERIALS AND METHODS

Samples collection and virus isolation: Samples were collected from cloaca swab of native chicken (*Gallus gallus domesticus*) that suspected infected by Lentogenic NDV from five live bird markets in Surabaya (Bratang, Rungkut, Keputran, Kapasan, and Wonokromo). Transport medium was consisted of 105,000 IU penicillin (0.35 ml), 105,000 IU Streptomycin (0.525 ml) and 0.5 ml of sodium chloride. Samples were stored in 4ºC refrigerator before inoculated into SPF embryonated eggs. All suspected samples were inoculated in 8 days old SPF embryonated eggs then incubated for five days in incubator (37ºC). Virus isolation was conducted according to manual instruction from OIE [3]. Candling observation was done once daily. During incubation period, death embryonated eggs were recorded then chilled (4ºC). In the end of incubation, embryonated eggs contained both death and live embryos were chilled (4ºC). They were processed into confirmation test.

HA and HI assays: All samples were processed into Haemagglutination assay (HA) to detect the presence of Haemagglutinin protein in isolated samples. Both assays were done used micro technic method in V bottom 96 well microplate. HA assay was conducted using 50 µl of 0.5% chicken erythrocyte [3]. All positive HA samples were processed into Haemagglutination Inhibition assay (HI) using 2^6 NDV antiserum titre to confirm that isolated samples contained NDV. Each sample was diluted into 4HAU before processed into HI assay. HI assay was conducted using β-method **[3]**. Positive NDV samples were processed into serial passages in SPF embryonated eggs incubated for five days. The passages were done for five times to observe the death time of infected embryo. Embryos death at <60 *hours post infection* (hpi), 60-

90 hpi, and >90 hpi were suspected as Velogenic, Mesogenic and Lentogenic NDVs respectively [17]. Suspected lentogenic NDV were processed into RNA extraction.

RNA extraction: Suspected Lentogenic NDV samples processed into RNA extraction according to manufacturer protocol of R&A-BLUE™ iNTRON (BIOTECHNOLOGY, 17501). Extracted RNA of each samples were stored in -80ºC.

One Step RT-PCR: One step RT-PCR was conducted according to manufacturer protocol of SuperScript® III One-Step RT-PCR System with Platinum®Taq DNA Polymerase (Invitrogen, 12574-026). Primer that used for one step RT-PCR was degenerated primer. PCR amplification was performed using following primers: forward primer 5'-GAG GTT ACC TCY ACY AAG CTR GAG-3' and reverse primer 5'-AGT CGG AGG ATG TTG GCA GC-3'. It was designed to amplify nucleotide number 4319-5005 of whole NDV genome (686 bp). 24 µl of RT-PCR master mix was added into each sample. The master mix was consisted of 12.5 µl of 2X RT Mix, 1 µl, respectively of primers, 8.5 µl NFW (DNase & RNase FREE, MP Biomedicals, 821932), and 1 µl SuperScript® III RT/Platinum®Taq Mix. One Step RT-PCR was run using thermo cycler machine (MJ Mini Personal Thermal Cycler, 1148). RT-PCR was arranged at 95ºC for *Lid on,* 94ºC predenaturation for 10 minutes, 94ºC of denaturation for a minute, 56ºC of annealing for a minute, 72ºC of extension for 2 minutes and 72ºC of extension prolong stage for 10 minutes. The cycles were repeated for 40 times. The product of PCR were stored in 4ºC. Product of RT-PCR was visualized using 1.5% agarose gel (UltraPure™ Agarose, Invitrogen, 15510-019). DNA marker for electrophoresis was 100-1,000 bp (OneMark DNA Ladder). Agarose gel electrophoresis machine (PowerPac Basic, BIO-RAD) was run at 100 V, 400 mA for 40 minutes. Agarose gel then observed under UV illuminator then purified for sequencing according to protocol of QIAquick® PCR Purification Kit (Qiagen, 28104).

Sequencing and molecular analyses: RT-PCR product was sequenced using ABI 310 xL GENETIC ANALYZER (Applied Biosystem Inc.). RT-PCR product was labeled using BigDye® Terminator Sequencing RR-100. The sequencer machine was arranged as; first rapid thermal ramp 96ºC for 10 seconds; second rapid thermal ramp 50ºC for 5 seconds, third thermal ramp 60ºC for 4 minutes. These cycles was repeated for 25 times. The samples was cooled in 4 ºC (fourth thermal ramp) until the samples had been ready to be precipitated. The samples were precipitated using 100 µl of absolute alcohol concentration. The sequence of nucleotides are symbolized as capital letters which are determined by International Union of Pure and Applied Chemistry (IUPAC). The sequence of nucleotides were analyzed using Biological Sequence Alignment Editor (BioEdit) version 7.0.5.3 and Molecular Evolutionary Genetics Analysis (MEGA) version 6.06. Isolated NDV were confirmed by comparing amino acid sequence in fusion protein cleavage site to Lentogenic NDV Lasota.

RESULTS AND DISCUSSION

Thirty seven cloacal swabs were collected from native chicken (*Gallus gallus domesticus*) that show clinical signs such as mild diarrhea, subclinical respiratory infection, and prostration [18]. There were 37 samples were collected. All samples and Lentogenic NDV positive control (Lasota) were inoculated into allantoic fluid of 8 days old SPF embryonated eggs. They were incubated and observed for five days. Allantoic fluid was harvested then processed into HA and HI assays. Positive HA assay was showed by agglutination of 0.5% chicken erythrocyte in the V bottom microplate. Ten suspected samples were showed positive HA assay (Table1).

All positive HA assay samples and positive control were processed into HI assay using 2^6 of NDV antiserum. Positive HI assay was showed by the absence of agglutinated erythrocyte in the bottom of the microplate until well number six. Five of ten of suspected samples showed positive HI assay (Table 2). Cross protection is occurred among Paramyxoviruses. It can be

mistaken with Avian Paramyxovirus serotype 3 (APMV3) and APMV7. But APMV3 and APMV7 never been isolated from chicken before [17] and they are not isolated from Indonesia. Commonly Indonesian raising chicken such broiler, layer, and native chicken. NDV firstly isolated in Indonesia around 1926 [26]. Since its first emerging, NDV or so called `tetelo' become endemic disease in Indonesia and cause annual outbreak [20, 26]. The samples were collected from native chicken (*Gallus gallus domesticus*) sold in bird market. Native chicken is traditionally reared in rural area which vaccination program is not conducted. This condition increases the possibility of natural infection of NDV. The aims of analysing lentogenic NDV is to know their mutation pattern as Lentogenic NDV has possibility to mutate into the virulence one. Thus it is very important to be observed.

Table 1: Titer of HA test of suspected Lentogenic NDV samples. Samples showed various titre

Samples	Titre of HA Test
NDVL1	2^6
NDVL2	2^6
NDVL3	2^9
NDVL5	2^4
NDVL6	2^6
NDVL7	2^9
NDVL8	2^5
NDVL9	2^9
NDVL10	2^8
NDVL11	2^9
LaSota	2^9

Table 2: Positive HI test samples means the isolated virus is NDV

Samples	HI titer
NDVL1	2^6
NDVL3	2^6
NDVL9	2^6
NDVL10	2^6
NDVL11	2^6
LaSota	2^6

Positive samples were observed in serial passages to select samples suspected Lentogenic NDV based on death time. Lentogenic NDV causes death of embryonated egg after 90 hours incubation [17]. All positive samples caused death of embryonated eggs after 90 hours post infection). It was supported the suggestion that isolated virus was Lentogenic NDV. But this suggestion compulsory to be confirmed by analysing amino acid at Fusion protein cleavage site of all samples. Suspected samples were processed into one step RT-PCR. RT-PCR product was visualized in 1.5% agarose gel, then it was observed under UV illuminator (Fig. 1).

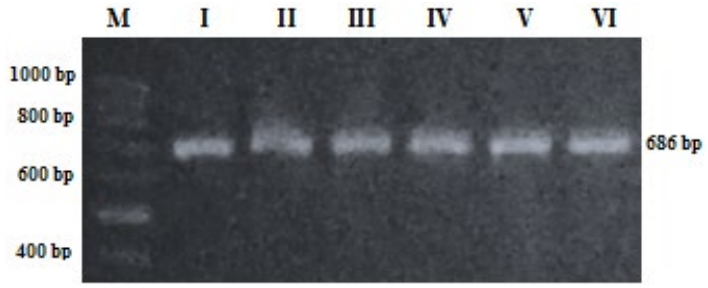

Figure 1: Agarose gel electrophoresis of suspected Lentogenic NDV samples. The band of all samples were located between marker for 600 bp and 700 bp. It means that primer amplified targeted region of whole NDV genome. M: 100 bp DNA ladder marker. Lane I: NDVL1. Lane II : NDVL3. Lane III : NDVL9. Lane IV : NDVL10. Lane V : NDVL11. Lane VI : LaSota (positive control).

RT-PCR product was processed into sequencing. Nucleotide sequence of the samples were analyzed using BioEdit version 7.0.5.3 and Molecular Evolutionary Genetics Analysis (MEGA) version 6.06. to confirm that isolated virus was Lentogenic. They were analyzed in region of fusion protein cleavage site. Amino acid sequence at fusion protein cleavage site of NDV was located at amino acid number 112-117. Lentogenic NDV has only single basic amino acid at amino acid number 112 and 115, and Leucine at amino acid number 117 [19]. Isolated samples showed identic sequence compared to Lasota/JF950510.1 (Fig. 2). It can be seen that amino acid sequence of the samples, marked with 'dot' and colored as same as amino acid of the reference sequence.

Figure 2: Confirmation of isolated samples by comparing their amino acid sequence of Fusion protein to Lasota/JF950510.1.

After confirming that isolated samples were Lentogenic NDV, product of RT-PCR that had been sequenced was aligned to Lasota/JF950510.1 (Genebank). The Nucleotide mutations were classified according to the type and their alteration of amino acid mutation. It was done to understand the effect of nucleotide mutation to amino acid sequence.

Sixteen nucleotide mutations occurred in NDVL1. They were occurred only on Fusion protein encoding gene. They were consisted of nine transitions mutations and seven transversion mutations. These caused some of amino acid alteration (Table 3).

Table 3: Nucleotide and amino acid mutations of NDVL1 only occurred on Fusion protein encoding gene

No.	Nucleotide mutation	Amino acid mutation	Type of mutation
1	A586G	I196V	Transition
2	G595A	G199S	Transition
3	G598A	I200V	Transition
4	G600C	-	Transversion
5	T618A	-	Transversion
6	C621G	-	Transversion
7	C623A	A208E	Transversion
8	A627G	-	Transition
9	A633T	-	Transversion
10	G636A	-	Transition
11	C639T	-	Transition
12	A642G	-	Transition
13	C644G	A215G	Transversion
14	T645C	-	Transition
15	G648A	-	Transition
16	A654T	Q218H	Transversion

Thirty six nucleotide mutations were occurred in NDVL3. They were consisted of twenty two transition mutations and fourteen transversion mutations. Six mutations were occurred at genome junction, while the rest of mutations were occurred at gene encoding Fusion protein (Table 4). Eleven mutations were occurred in NDVL9. They were consisted of eight transition mutations and three transversion mutations. All mutations were occurred at gene encoding Fusin protein (Table 5). Nucleotide and amino acid mutations which found on NDVL10 and NDVL11 were particularly same. They were consisted only of transition mutations. One mutation occurred at gene encoding Matrix protein while four mutations were occurred at gene encoding Fusion protein (Table 6).

According to the observation, 89.2% mutation occurred on Fusion protein encoding gene. Fusion protein is the major determinant protein in genotyping and virulence classification of

Newcastle Disease Virus. It is noted that Fusion protein is immunogenic protein and target of antibody-antigen neutralization [16]. Among samples, nucleotide and amino acid mutations on genome junction occurred only on NDVL3 (Table 5). Intergenic region of NDV extend between each protein encoding gene. The modification of its domain is capable to increase the virulence of NDV [24].

Table 4: Nucleotide and amino acid mutations of NDVL3 occurred on genome junction (red color) and Fusion protein encoding gene (black color)

No.	Nucleotide mutation	Amino acid mutation	Type of mutation
1	C102G	-	Transversion
2	A118C	K4Q	Transversion
3	T134C	L45P	Transition
4	A140T	Y47S	Transversion
5	G168A	-	Transition
6	T205C	-	Transition
7	C213T	-	Transition
8	C215T	S72k	Transition
9	C216T		Transition
10	C234T	-	Transition
11	A274G	I92V	Transition
12	T282C	-	Transition
13	G299C	C100S	Transversion
14	A339G	-	Transition
15	T342C	-	Transition
16	T357A	-	Transversion
17	C372A	-	Transversion
18	C387T	-	Transition
19	A405G	-	Transition
20	T414G	-	Transversion
21	G426A	-	Transition
22	T453C	-	Transition
23	G456A	-	Transition
24	C504T	-	Transition
25	T582C	-	Transition
26	G595A	G199S	Transition
27	G600C	-	Transversion
28	T618A	-	Transversion
29	C621G	-	Transversion
30	A627G	-	Transition
31	A633T	-	Transversion
32	G636T	-	Transversion
33	C639A	-	Transversion
34	C646G	L216V	Transversion
35	G648A	-	Transition
36	A663G	-	Transition

Table 5: Nucleotide and amino acid mutations of NDVL9 only occurred in Fusion protein encoding gene

No.	Nucleotide mutation	Amino acid mutation	Type of Mutation
1	T582C	-	Transition
2	G595A	G199S	Transition
3	G598A	V200I	Transition
4	G600C		Transversion
5	T618A	-	Transversion
6	C621G	-	Transversion
7	A627G	-	Transition
8	A633T	-	Transversion
9	G636A	-	Transition
10	C639T	-	Transition
11	A641G	-	Transition
12	G648A		Transition

Table 6: Nucleotide and amino acid mutations of NDVL10 and NDVL11 occurred on gene encoding Matrix protein (red) and Fusion protein encoding gene

No.	Nucleotide mutation	Amino acid mutation	Type of mutation
1	A23G	E8G	Transition
2	T582C	-	Transition
3	C621T	-	Transition
4	G636A	-	Transition
5	A642G	-	Transition

NDV is endemic viral disease in many regions include Indonesia [20]. It may be caused by the rise of poultry density but unfollowed by good biosecurity management [21]. Lentogenic NDV can be isolated from native chicken (*Gallus gallus domesticus*) in live bird markets. To define isolated NDV is Lentogenic strain, several methods must be done such as observation of death time and analyzing sequence of amino acid at cleavage site of Fusion protein. RNA viruses are noted easily to mutate [2].

Some of nucleotide mutations that occurred caused amino acid mutations, but some of them are not. Mutations did not occurred in cleavage site of Fusion protein (nt 558-576) means the virulence of samples compared to reference sequence are same. The mutations that occurred may not effect the virulence of the samples. Even though it did not occur in this research, certain mutation in cleavage site of fusion protein transform the Lentogenic NDV into the virulence one [16]. Polymerase protein of RNA virus is characteristically for its low fidelity which lead RNA viruses are easily to mutate. These mutations may form quasispecies Newcastle Disease Virus [22]. Quasispecies is viruses genetically linked as the result of genetic variation process, competition, and selection. They are non identic but belongs to same lineage [2]. Each mutant in quasispecies is genetically linked [23]. It needs further research to understand concept of quasi species in Newcastle disease virus.

NDVL10 and NDVL11 are suspected to be one species since it is isolated from same live bird market. Among the samples, NDVL10 and NDVL11 have the least nucleotide and amino acid mutations. Furthermore, the position of the mutations are same. According to this finding it is suggested that these samples are `Lentogenic NDV like vaccine. It could be found in wild birds too [25].

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