CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS LENTOGENIC STRAIN INFECTED NATIVE CHICKENS FROM SURABAYA, INDONESIA

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ORIGINAL ARTICLE

CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS LENTOGENIC STRAIN INFECTED NATIVE CHICKENS FROM SURABAYA, INDONESIA

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ABSTRACT

This study aimed to characterize the lentogenic NDV isolate from native chickens in Surabaya, Indonesia. Thirty seven samples of cloacal swabs from infected native chickens were collected, and pathotypes were characterized using Mean Death Time (MDT) analysis; six isolates were found to be lentogenic. Lentogenic NDV isolates were then analyzed via RT-PCR, using primers specific for cleavage site of fusion protein (F). PCR product was sequenced and analyzed using Epitope Prediction Tools (IEDB) Analysis Resource to determine the epitopes. The results showed some shifts in nucleotides, but no change was observed in amino acids. Five samples were found with similar sequence in the cleavage site, except NDV isolated from sample Ck/sby27, which had a different amino acid sequence, "RRQKRFI." Epitope characterization specific for T-cell NDV was found in Ck/sby27 at position 42-142, with highest real score in epitopes CLDYLQLVY, SIDGRPLAA, and TAEQITAAA. These findings reinforce the assumption that the original NDV lentogenic strain is of wild origin and is still circulating in the environment. Therefore, NDV isolate Ck/sby27 can be used in developing vaccines in the future.

Key words: Indonesia, lentogenic strain, native chicken, NCD, T-cell epitopes

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INTRODUCTION

Newcastle Disease Virus (NDV) is a highly contagious infectious disease. Although vaccination has been employed in its control, it remains widely spread across many regions in the world and is still endemic in 57% countries with developing poultry industries (Czegledi *et al*., 2006). NDV can be transmitted mostly from different wild avians, including wild birds and waterfowls (Ganar *et al.*, 2014). These avians are carriers of exotic NDV and can come into contact with native poultry farms, given the traditional farming practices where unvaccinated chickens are typically allowed to roam freely in open spaces. For this reason, this study seeks to find local and original NDV lentogenic strain isolates to be used as seeds for vaccine development.

NDV can be classified into five pathotypes based on clinical signs: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic (Aldous and Alexander, 2001; Muelemans *et al*., 2002). Furthermore, it is defined according to amino acid sequence in F_0 cleavage site. The presence of multiple basic amino acids in the cleavage site indicates its pathogenicity (Smietanka *et al.*, 2006). The virus has caused panzootics around the world, also termed as virulent NDV (vNDV). The first panzootic, caused by

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viruses belonging to genotypes II, III and IV of class II, occurred between 1920s and 1960s (Alexander, 2000; 2004; Miller *et al.*, 2009a). The second panzootic, caused by genotype V virus, occurred in the late 1960s in Europe (Lomniczi *et al.*, 1998). Meanwhile, a virus under subgenotype VIb was responsible for the third panzootic that infected pigeons in the 1980s (Kaleta *et al.*, 1985; Miller *et al.*, 2009a). The fourth panzootic, caused by viruses belonging to genotype VII, was a massive one, spreading throughout Asia, Africa, Europe and South America (Moya *et al.*, 2000; Perozo *et al.*, 2012). Further, a new subgenotype VIIi isolated from Israel, Indonesia and Pakistan was deemed to have lit the fifth panzootic. In Indonesia, NDV was first isolated in 1926 then became endemic since (Xiao *et al.*, 2012). OIE reported 1,500-8,000 cases of NDV infection in Bali in 2007 (Adi *et al.*, 2010). This saw a succession of 50-80% mortality in poultry from 2009-2010 (Xiao *et al.*, 2012) and 2011-2012 (Miller *et al.*, 2015).

In Indonesia, NDV infection incurred a massive loss of approximately 142 billion rupiahs due to decrease in production and hefty management costs. The virus is still endemic in Indonesia (Ronohardjo and Halim, 1995). Consequently, routine vaccination has been implemented but such attempts had poor results (Sharif *et al.*, 2014). This may be attributed to inadequate protection against vaccine strains used in the poultry industry and poor biosecurity (Chukwudi *et al.*, 2012). Likewise, it is also possible that NDV strain circulating in the environment is a different pathotype or strain, or exotic birds may be responsible for spreading the infection (Chakrabarti *et al.*, 2007). Vaccine strains are of genotype II, which has different genetic properties than genotype VII and VIII that are causing sporadic outbreaks (Forrester *et al.*, 2013). This dissimilarity might only confer minimal protection to the poultry industry (Dharmayanti *et al.*, 2014; Ganar *et al.*, 2014).

This study was conducted to determine the lentogenic original NDV strain from native chickens. This was chosen since native poultry farms practice traditional farming, wherein typically unvaccinated chickens live in open spaces, which then provide contact with outside fauna, such as wild roaming birds. The outcome of this study can be used as a basis for future vaccine development.

MATERIALS AND METHODS

Sample collection

NDV isolates were obtained from cloacal swabs of 37 native chickens in Surabaya, Indonesia. This study was approved by the Institutional Animal Care and Use Committee, with ethical clearance No. KE 105.07.2018 under the guidance of the Ethical Clearance Commission of the Faculty of Veterinary Medicine, Universitas Airlangga.

Mean death time

To determine the pathotypes, mean death time (MDT) adopted from the Organization for Animal Health (OIE, 2012) was used. Fresh, sterile phosphate buffer saline (PBS) was used to dilute allantoic fluid by ten-fold dilution between 10-5 and 10-9. For inoculation into the cavity of each five 10-day old embryonated SPF chicken eggs, 0.1 ml sterile PBS was used and then incubated at 37°C. Each egg was observed twice daily for seven days; dead embryos were recorded. MDT denotes the mean time in hours that would kill all inoculated embryos, and the minimal lethal dose is the highest virus dose that would eliminate all inoculated embryos (OIE, 2012).

Intracerebral pathogenicity index

The method from Organization for Animal Health (OIE, 2012) was used to determine intracerebral pathogenicity index (ICPI) (Table 1). Allantoic fluid of harvested virus was diluted to 1:10 using sterile PBS. The day-old native chickens were inoculated intracerebrally with 0.05 ml diluted virus, examined every 24 h for eight days, and given scores (0: normal, 1: sick, 2: dead). ICPI is the mean score per bird per observation over the eight-day period (OIE, 2012; Parotto *et al.*, 2012; Garcia, *et al.*, 2013; Mirza *et al.*, 2013; Nakamura *et al.*, 2014).

RNA extraction

Harvested allantoic fluid samples were then used for the characterization of F gene

Table 1. Intracerebral pathogenicity index (ICPI) values of NDV isolates: mean death time (MDT, h) of chick embryos infected with one minimum lethal dose and ICPI in day-old chicks.

No.	Sample	MDT (hpi)	ICPI
1	Ck/sby10	120	0.12
2	Ck/sby11	120	0.12
3	Ck/sby26	120	0.25
4	Ck/sby27	116	0,37
5	Ck/sby30	120	0.12
6	Ck/sby33	120	0.25
7	LaSota (positive control)	120	0.12

coding protein to find the original NDV isolate lentogenic strain. First, 700 µl of trizol (R&A-blue™, Intron Biotechnology) was added into 1.5 ml microtube containing 300 µl allantoic fluid, then mixed in room temperature for 1 min under BSC II and added with 200 µl absolute chloroform. After mixing using vortex (Advance Tme-21 test tube mixer) for 15 sec, the mixture was centrifuged at 13,000 rpm at 4°C for 10 min (Scientific Sorvall™ Legend™ Micro 17R, Thermo Fisher Scientific). After aqua part was collected, solution was transferred into a 1.5 ml microtube, added with 400 µl of absolute isopropanol, left at room temperature for 10 min, and centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was carefully discarded, and pellet is dried using safety cabinet fan, then added with 1 ml of 75% ethanol. After centrifugation at 7,500 rpm for 1 min, the supernatant was carefully discarded, then dried using a safety cabinet fan for approximately 5 min, and added with 15 µl of RNAse Free Water (DNase & RNase FREE, MP Biomedicals). Solution was incubated at 55°C (J-100, JISICO) for 10 min, and after that RNA was directed into cDNA or stored at -70°C. Finally, concentration was measured with a fluorometer (Qubit™ Fluorometer, Invitrogen) (Ahmadi *et al*., 2013).

One step RT-PCR

Reverse Transcriptase-PCR was done to amplify the genome of RNA viruses. Twenty-four µl of one step RT-PCR master mix (SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase, Invitrogen) was placed into 100 µl microtube, added with 1 µl of RNA, mixed by tipping, and spinned (BIO-RAD). The tubes were transferred into a thermocycler (MJ Mini™ Personal Thermal Cycler, Bio-Rad). Primers were used for amplified cleavage site of Newcastle disease virus fusion protein gene (Forward, 5'-GAGGTTACCTCTACTAAGCTG-3', and Reverse, 3'-AAGTCGGAGGATGTTGGCAGC-5').

The thermocycler was set at the following conditions: 95°C of Lid on, pre-denaturation at 94°C for 10 min, denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension stage at 72°C for 2 min, and another extension at 72°C for 10 min. Denaturation took forty cycles. Amplicons (product of PCR) were stored at 4°C.

RT-PCR product gel electrophoresis

Gel electrophoresis was used to detect and visualize the amplified RT-PCR product. 1.5% agarose gel was placed into the electrophoresis machine (PowerPac™ Basic Power Supply, Bio-Rad) and the medium was adjusted accordingly. Then, 7 µl of blue Juice master mix solution (10 × BlueJuice™ Gel Loading Buffer, Invitrogen) was put on a parafilm where 1 µl of amplicon was added. The solution was mixed and samples were transferred to the wells. Then, 8 µl of DNA marker solution (OneMARK 100 DNA LadderDM101-100, GeneDireX Inc.) was pipetted into well number 1. Finally, electrophoresis was run at 100 V, 400 mA for 40 min, and the gel was checked under UV illuminator.

Sequencing

Sequencing was done to analyze the nucleotide sequence in the amplified RT-

PCR product. The RT-PCR product was purified using QIAquick® PCR Purification Kit (Qiagen) according to the protocol. The following components were placed into a 100 µl with dye-labelled 1.5 µl BigDye® Terminator Sequencing RR-100: 5 µl of 5× sequencing buffer, 5 µl of purified amplicon, 1.5 µl of forward and reverse primers and 7 µl of nuclease free water (Promega). These were mixed, spinned carefully and transferred into a thermal cycler (ABI 3130xl Genetic Analyzer, Applied Biosystems Inc.) set at 96ºC to heat the tubes for 3 min. The cycles were set at the following conditions: first rapid thermal ramp at 96ºC for 10 sec, second rapid thermal ramp at 50ºC for 5 sec, and third thermal ramp at 60ºC for 4 min, repeating the cycle 25 times. Fourth thermal ramp at 4ºC was kept until the samples were ready to be precipitated. Precipitation for each tube was done using 100 µl of 100% alcohol concentration. The result of sequencing was presented as symbols determined by the International Union of Pure and Applied Chemistry (IUPAC). Sequence result was used to edit the samples of NDV lentogenic strain. After combining and editing, sequence samples were trimmed. In this study, only fusion protein gene was used. Trimming was done by aligning the samples to the whole lentogenic genome at nucleotides 4319-5005. The fusion protein gene has 687 bp. Reference sequence was placed at the first row, followed by the sequence of samples. Alignment was run using Crustal W Multiple Alignment program within the accessory application menu of BioEdit ver. 8.0 software. After the alignment, the regions of the matrix protein gene and genome junction sequence were cut and sequence was analyzed using MHC class-I binding peptide predictions from the IEDB Analysis Resource.

RESULTS AND DISCUSSION

Pathogenicity test of the NDV isolate of native chickens

Thirty-seven samples of cloacal swab from native chickens were inoculated into chorioallantoic fluid of ten day-old embryonated chicken eggs. Each sample was inoculated into three embryonated eggs. Samples were confirmed for NDV according to their HI titers and were biologically characterized. MDT scores ranged from 116 h to 120 h, and ICPI scores of all samples were 0.12 except Ck/sby27, which had an ICPI score of 0.37. Based on these data, six samples were considered lentogenic. These were then analyzed using RT-PCR and specific primers to explore the fusion protein gene (Fig.).

Characterization of F protein gene

After purified DNA was used for sequence analysis, nucleotides were translated into amino acids to characterize the epitopes. The sequence of amino acids were compared to the positive controls and reference isolates (LaSota and Kumarov strains), showing some mutations in different regions. Alignment of amino acid sequence in between samples and references showed that most samples did not manifest significant mutation, except for Ck/sby27 isolate. Ck/sby27 isolate has mutations throughout the nucleotide residues of 418 to 664, which plays an important role in the mutation throughout amino acid residues of 136 to 208.

Partial sequence and restriction enzyme cleavage site of the fusion protein gene were used to analyze and compare the difference of six Newcastle disease virus isolates from native chickens with that of LaSota strain genotype II. Overall, isolates have amino acid sequence similarities except Ck/sby 27. It is still unclear, however, whether the isolate was infected with the original NDV or NDV vaccine, since the latter is an NDV genotype II, which has caused sporadic outbreak in the US (Czegledi *et al.*, 2002).

Epitope prediction of NDV fusion (F) protein T cells

Epitope prediction of the T cells of F protein specific of NDV isolate nucleotide sequence was translated into amino acid sequence using BioEdit ver. 8. software and MHC class-I binding peptide predictions from the IEDB Analysis Resource. This produced peptide fragments with their position and sequence.

Table 2 shows the analyses of five epitopes of LaSota strain T cells, wherein three epitopes

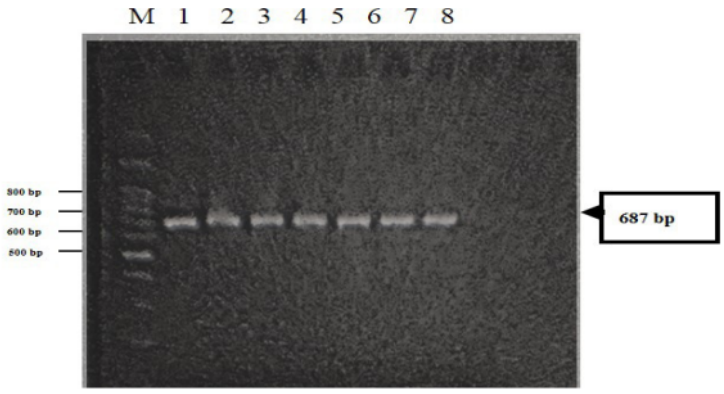

Fig. RT-PCR analysis of NDV isolates fron native chickens in Surabaya, Indonesia. (M) marker; (1-6) NDV samples; (7) LaSota as positive control; and (8) PBS as negative control.

are deemed vital to immune response: residue 42 with sequence CLDYLQLVY, residue 8 with sequence GKEHTLAKY, and residue 104 with sequence SIDGRPLAA. Epitope prediction was performed on the Ck/sby27 isolate (Table 3). It was found that compared to the epitope prediction on LaSota strain, only two were important epitopes with sequences CLDYLQLVY and SIDGRPLAA. Unlike LaSota strain, no epitope was found at position 8 with sequence GKEHTLAKY. The Ck/sby27 have similarities with Ck/sby10, Ck/sby11, Ck/sby26, Ck/sby30 and Ck/sby33. All of the isolates have CLDYLQLVY sequence with a log score 4.8283 and SIDGRPLAA with a log score 0.9163.

NDV has a single stranded RNA (ssRNA) with at least 10 genotypes worldwide. Although not all NDV genotypes have spread throughout Indonesia, many have circulated, including the virulent genotype VII that incited sporadic outbreak in sectors 1 to 4 of the poultry industry.

This study explored NDV genotype II and lentogenic pathotype in native chickens to know whether these strains are still circulating in the environment. After all samples were analyzed for their pathotypes, MDT showed that all isolates ranged from 116-120 h, while ICPI ranged from 0.12-0.37. Results clearly show that NDV lentogenic strain is still present in the environment despite the country's efforts to ward off the virus through routine vaccination within the poultry industry. It is important to consider, though, that endemic areas in the country remain a potential source of NDV, and that being archipelagic, the country hosts many migrating birds, which can potentially spread the virus. NDV being an RNA virus makes it an important pathogen, and this has substantial implications to commercial poultry worldwide, as it may cause outbreaks and trade barriers (Alexander *et al*., 2004). Pathotyping of NDV from native chickens becomes extremely important because of the substantial variation in the virulence of different NDV isolates and the widespread use of live vaccines.

After six samples were determined using RT-PCR, F protein coding gene in cleavage site region was determined to have 687 bp using specific primers. Then, characterization of amino acid sequence showed that all samples were similar. The presence of NDV isolates from native chickens depends on the geographic and temporal proximity. They are related to virus present in the poultry because lentogenic strains were used as vaccines in different levels of the poultry industry (Miller *et al*., 2009b). These might be due to viral evolution (Miller *et al*., 2009b), but there is one example, Ck/sby27, which produced a different amino acid sequence in the cleavage site region. Its amino acid sequence was RRQRFI and reference sample was GRQGRI (Table 4).

These results have important roles in characterizing the peptide glycoprotein or fusion protein (Horvarth *et al*., 1992; Heiden *et al*., 2014). The mutation in Ck/sby27 can be caused by many factors: mixed infection

Table 2. Epitope prediction of F protein from NDV LaSota strain of vaccine isolated from native chicken in Surabaya, Indonesia.

Epitope prediction for T cell LaSota strain					
	Threshold for 4% with score: -0.693		Highest score achievable by any peptide on log scale: 12.324		
Rank	Sequence	Position	Real score	Log score	% highest on log scale
1	CLDYLQLVY	42	125	4.8283	39.18
2	GKEHTLAKY	8	4.5	1.5041	12.20
3	SIDGRPLAA	104	2.5	0.9163	7.44
4	RVEDSGSRL	59	0.625	-0.1054	0
5	TGDKAVNIY	118	0.25	-0.4700	0

Table 3. Epitope prediction of F protein from NDV Ck/sby27 isolated from native chicken in Surabaya, Indonesia.

Sample	Cleavage site of amino acid F protein						
	112	113	114	115	116	117	118
LaSota /JF950510/GenBank reference	G	R	Q	G	R	L	I
Positive LaSota isolate/vaccine reference	G	R	Q	G	R	L	I
Ck/sby10	G	R	Q	G	R	L	I
Ck/sby11	G	R	Q	G	R	L	I
Ck/sby26	G	R	Q	G	R	L	I
Ck/sby27	R	R	Q	K	R	F	I
Ck/sby30	G	R	Q	G	R	L	I
Ck/sby33	G	R	Q	G	R	L	I

from the environment and vaccine strains and spill over from strains of other species, microenvironment and macroenvironment. Failure to resolve the exact cause of this mutation can lead to an outbreak (Garcia *et al*., 2013; Miller *et al*., 2015).

NDV lentogenic strain has been used as a live vaccine material worldwide, therefore this strain is still highly circulating in the environment. Such strain was used since NDV strain has cross protection against different pathotypes, like mesogenic and velogenic. This response shows that lentogenic strains are vital to vaccine development. Specifically, low virulent NDV strains can induce the production of cellular antibodies, conferring protection against NDV. In turn, this also induces neutralizing antibodies to act against viral fusion glycoprotein and hemagglutinin protein, which play an important role in immune response (Kapczynski *et al*., 2013). In this study, only one sample showed different properties of amino acid. Therefore, it is yet to be determined whether an original wild infection is likely to occur relative to other infective strains.

Further characterization to predict specific T cell response-inducing epitope using MHC class-I binding peptide prediction server found two to three kinds of epitope for every isolate. The epitopes with high log score of 4.8283 has the sequence CLDYLQLVY, while the epitope with lower log score of 0.9163 has the sequence SIDGRPLAA. These isolates have two epitopes that resemble two out of the three epitopes of LaSota strain, and these might be the epitopes that overlapped between MHC II and MHC I (Osman *et al*., 2016).

It can be concluded that although these isolates hold similarities with LaSota strain, they are likely to be of different strains. Kim *et al*. (2007) have already found the original

Table 4. Amino acid in cleavage site of fusion protein of NCD isolated from native chicken in Surabaya, Indonesia compared to genotype II of GenBank and vaccine reference.

Sample	Cleavage site of amino acid F protein						
	112	113	114	115	116	117	118
LaSota /JF950510/GenBank reference	G	R	Q	G	R	L	I
Positive LaSota isolate/vaccine reference	G	R	Q	G	R	L	I
Ck/sby10	G	R	Q	G	R	L	I
Ck/sby11	G	R	Q	G	R	L	I
Ck/sby26	G	R	Q	G	R	L	I
Ck/sby27	R	R	Q	K	R	F	I
Ck/sby30	G	R	Q	G	R	L	I
Ck/sby33	G	R	Q	G	R	L	I

NDV isolate from waterfowls and shorebirds, and this might also be the case in this study.

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