Original article

MOLECULAR CHARACTERISATION OF NEWCASTLE DISEASE VIRUS EXOTIC ISOLATE IN EAST JAVA, INDONESIA

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Summary

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Newcastle disease virus (NDV) is *ss*RNA paramyxovirus causing clinical signs, varying from subclinical infections to 100% mortality in infected chickens. Haemagglutinin-neuraminidase (HN) protein has an important role related to infection and pathogenesis, therefore, the protein was characterised in this study. Samples were collected from 45 cloacal swabs of native chickens. They were isolated by inoculating in specific pathogen-free embryonated eggs. Molecular detection of NDV was done by reverse transcriptase polymerase chain reaction (RT-PCR) encoding HN protein. RT-PCR for HN gene of NDV generated DNA fragments sized 503 bp, which were then sequenced using ABI Prism. The results have shown that virus isolates were mostly lentogenic and might contribute to outbreak in East Java, Indonesia. Based on this fact, NDV infected native chickens can act as reservoir and contribute to outbreak in the poultry. Our study provides baseline information on genetic characteristics of NDV circulating in East Java and serves as a basic work for further research.

Key words: gene coding HN, NDV*,* native chicken, new variants, RT-PCR

INTRODUCTION

Indonesia is an endemic area of Newcastle disease virus (NDV), which is spread all over Indonesia. The disease, which is known to have infected over 200 bird species, is spreading rapidly, both morbidity and mortality of NDV infection can reach 100% (OIE, 2012). NDV outbreaks during 2009–2010 (Xiao *et al*., 2012) and during 2011–2012 (Dharmayanti *et al*., 2014; Miller *et al*., 2015) with 70−80% mortality of poultry have caused a total loss of approximately US$ 10.011 million due to the decrease in production and management cost (Dharmayanti *et al*.,

2014). Preventions such as routine vaccination and proper management had been done but the implementation of these preventions is still not enough to preclude the outbreak of NDV (Dimitrov *et al.*, 2017).

Prophylactic measures can be done through vaccination. Vaccination against ND is done to control the impact of both low virulent and virulent NDV infections (Su *et al.*, 2018). Vaccines are many types; live attenuated vaccine based on highly immunogenic NDV strains of low or intermediate virulence, and inactivated vaccines (Bello *et al.*, 2018). It is noted that the NDV strain circulating in Indonesia is distinct compared to the commercial vaccine used. NDV strains in Indonesia belong to class II genotypes I, VI, and dominantly VII (Dharmayanti *et al.*, 2014) while the vaccine virus that is conventionally used is LaSota strain from class II genotype II. Based on a study of Xiao *et al.* (2012), analysis of the serologic response to the vaccination and challenge showed that vaccinated birds produced higher HI and neutralising antibody titres against genotype-matched viruses than genotype-mismatched virus therefore genotype homology between vaccine virus and the infecting NDV has an important role to reduce virus shedding in the environment (Miller *at al.*, 2013).

The aim of this study was to perform a molecular characterisation of NDV from native chickens based on the HN protein coding gene. This is very important in order to develop new vaccines.

MATERIALS AND METHODS

Sample collection

Newcastle disease virus (NDV) isolates were gathered from cloacal swabs of forty-five female native chickens aged 14–15 weeks weighing 1.5−2 kg from several regions of East Java, Indonesia. The research was approved by IACUC with the ethical clearance No. KE 105.07.2018 under guidance of Ethical Clearance Commission Faculty of Veterinary Medicine, Airlangga University.

Mean death time (MDT)

To determine the biological characteristics of ND viruses based on pathotype the OIE standard was used, briefly, fresh sterile phosphate buffered saline was used for serial dilution of allantoic fluid by tenfold dilution between 10^{-5} and 10^{-9} for inoculation of 0.1 mL into cavity of each of five 10-day-old embryonated SPF chicken eggs and then incubated at 37 $^{\circ}$C. Meanwhile each egg was investigated twice daily for seven days, and embryo deaths were recorded. MDT was the mean time in hours for the minimal lethal dose to kill all of the inoculated embryos, and the minimal lethal dose was the highest virus dose causing all of the embryos inoculated with virus with a specific dilution to die (OIE, 2012).

Intracerebral pathogenicity index (ICPI)

To determine the biological characteristics of ND viruses based on the pathotype, the OIE standard was used. Allantoic fluid of harvested virus was diluted to 1:10 using sterile PBS. Then it was inoculated intracerebrally to day-old chickens at 0.05 mL diluted virus and then examined every 24 h for eight days. Birds were scored with 0 if normal, 1: if sick and 2: if dead. ICPI was the mean score per bird per observation over the eight-day period (OIE, 2012; Nakamura *et al.*, 2014).

RNA virus extraction

RNAs of the viruses were extracted from allantoic fluids using Trizol LS reagen

(Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Extracted RNA was stored at −80 ºC.

Reverse transcriptase polymerase chain reaction (RT-PCR)

In each reaction 12.5 µL of reaction mix solution, 1 µL of each reverse and forward primers (forward primer 3- AGTTAGCC AGTTGCGTTAG-5, and reverse primer 5-AACCTGATCCTGTATAGGC-3 with length DNA 503 bp with concentration 20 pmol/L), 2 µL of RNA, 1 µL of ssIII/ Platinum Taq Polymerase (SuperScript ® III One-Step RT-PCR System with Plati-num® Taq DNA Polymerase, Invitrogen by Thermo Fisher Scientific) were added. Finally nuclease free water was added until the volume reached 25 µL. The thermocycle machine was set at 95 ºC Lid on, 94 ºC for predenaturation stage for 10 mins, 94 ºC for denaturation stage for a minute, 57 ºC for annealing stage for a minute, 72 ºC for extention stage for 2 minutes and 72 ºC for final extension stage for 10 minutes (MJ Mini ™ Per-sonal Thermal Cycler, Bio-Rad). The cy-cles for the PCR were repeated 40 times in denaturation stage. The amplicons (product of PCR) were stored at 4 ºC.

RT-PCR products were visualised by agarose gel electrophoresis at 100 V for 60 min (Electrophoreses Machine, Power-Pac™ Basic Power Supply, Bio-Rad). The presence of marker and PCR product bands was observed under UV light.

Sequencing

The PCR product was purified using QIAquick® PCR Purification Kit (Qiagen) and labelled after the opurificai-ton. BigDye® Terminator Sequencing RR-100 (1.5 µL) was put into a 100 µL tube and 5 µL BigDye® Terminator 5× sequencing buffer, 5 µL of purified ampli-

con, 1.5 µL of forward and reverse prim-ers and 7 µL of nuclease free water (Free Water, Promega) were added into the tube, mixed then spinned down carefully.

Tubes were transferred into a thermal cycler (ABI 3130 xL GENETIC ANALYZER, Applied Biosystem Inc by Thermo Fisher Scientific). The result of sequencing was shown as symbols that was determined by International Union of Pure and Applied Chemistry (IUPAC).

Molecular analysis

The sequence of nucleotides was analysed using Biological Sequence Alignment Editor (BioEdit) version 7.0.5.3 (BioEdit software, Ibis Therapeutics) and Molecu-lar Evolutionary Genetics Analysis (MEGA) (Tamura *et al.*, 2013). Both for-ward and reverse sequences were com-bined using MEGA version 6.06 then ed-ited by using BioEdit version 7.0.5.3. Reference sequence (GenBank) was used to edit the samples was NDV LaSota. Then the homology analysis was done by comparing the partial haemagglutinin-neuraminidase protein of the isolated virus strain compared to Indonesian isolates submitted in GenBank. Reference se-quence can be accessed in GenBank. The homology was measured using global alignment (Needleman-Wunsch algo-rithm) of two nucleotides sequences via NCBI. To predict the B cell epitope, methods provided by immune epitope database (http://toolseidb.ofg/bcell/) with default threshold BepiPred value of 0.35 were used (Anayet *et al.*, 2013).

RESULTS

Biological characterisation of isolated NDV

Five out of forty-five samples showed positive results of HA. Those samples we-

Table 1. Biological characterisation of NDV isolated in Surabaya

NDV Isolate	Location	Pathotype	MDT (hpi)	ICPI
Sby-Ck01/13	Surabaya	Lentogenic	100	0.12
Blt-Ck02/13	Blitar	Lentogenic	120	0.12
Blt-Ck03/13	Blitar	Lentogenic	110	0.25
Sby-Ck35/16	Surabaya	Lentogenic	120	0.12
Pas-Ck36/16	Pasuruan	Lentogenic	100	0.37
Positive control	Surabaya	Lentogenic	120	0.12

Note: Mean death time in 9-day-old SPF embryonated chicken eggs (hours) (velogenic <60, mesogenic 60–90, lentogenic> 90). Intracerebral pathogenicity index in 1-day-old chickens (lentogenic <0.7, mesogenic 0.7–1.4, velogenic 1.4–2.0).
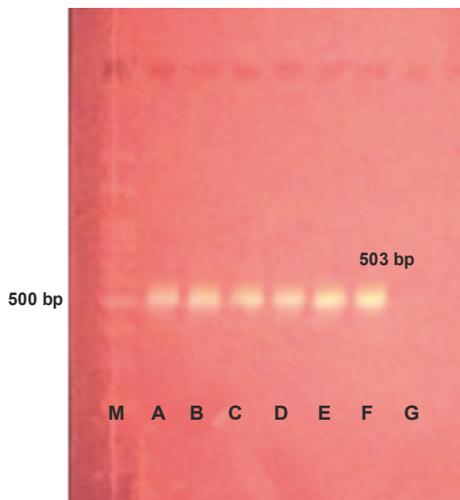
Fig. 1. The RT-PCR amplification results of the HN gene of NDV. Lane M: marker DNA ladder 100 bp. Lanes B: Sby-Ck01 (503 bp); C: Blt-Ck02 (503 bp); D: Blt-Ck03 (503 bp), E: Sby-Ck35 (503 bp), F: Pas-Ck36 (503 bp) are samples. Lane A: positive control (503 bp); Lane G: negative control.

re Sby-Ck01/13, Blt-Ck02/13, Blt-Ck03/13, Sby-Ck35/16 and Pas-Ck36/16. Further analysis of samples using haemagglutination inhibition assay was done to make sure that isolates were NDV, using specific sera provided by VETMA, Surabaya. Based on biological characterization, MDT was about 100–120 hpi and ICPI was about 0.12–0.37 (Table 1). The results have shown that all isolates were lentogenic strain. RT-PCR method with primers that are specific to lentogenic strains has also shown positive results. RT-PCR products visualised in 1.5% agarose gel under UV transilluminator was shown on Fig. 1.

Homology analysis

Based on the results of homology analysis of isolates, Sby-Ck01/13, Blt-Ck03/13, Sby-Ck35/16, and Pas-Ck36/16 had high homology, but Blt-Ck02/13 did not have high homology. Complete data are shown in Table 2.

Phylogenetic relationship of NDV from East Java

The relationship between positive samples obtained in this study and other NDVs available in GenBank was illustrated using a phylogenetic tree of the partial HN gene sequence of the 5 sequences obtained in the study and other GenBank sequence of NDV (Fig. 2). From the tree, Pasuruan isolate (Pas-Ck36/16) was different compared to other isolates in East Java, Indonesia. The Pasuruan isolate belonged to class I NDVs – low-virulence strain.

Epitope prediction of B cell

The B cell epitope is a specific antigen region that has a high interaction with B cell lymphocyte. As a result, B cell could produce the antigen-specific antibody and memory B cell. According to epitope predictions, samples that had high number of epitope were Sby-Ck01/13 (Table 3) and Pas-Ck36/16 (Table 4).

DISCUSSION

Vaccination is the most important way to prevent NDV infections (Wang *et al*., 2015). Many types of vaccines have been used, but NDV circulating in the field are different from the viruses used for vaccine causing outbreaks (Dimitrov *et al*., 2017). In addition, exotic NDV can also infect poultry in Indonesia. Therefore, it is very important to find the new variant and original isolate from native chicken to develop new vaccines.

In this study, we have analysed variations in the NDV genome in Indonesia, which have caused outbreaks in some poultry in the country. The poultry vaccine used in Indonesia comes from various countries and different strains, causing different viral properties. The HN protein of NDV has responsibility to induce antibody, as well as attachment between the outer membrane protein of virus and cell receptors (Absalon *et al*., 2019).

Five NDV isolates obtained from an outbreaks in East Java, Indonesia, were characterised to determine the pathotype of the virus. Pathogenicity tests showed that all isolates were lentogenic strains. All isolates demonstrated some pathogenicity in embryonated eggs and one-day old chicken. The results have shown that native chicken may not only be a natural reservoir of NDV, but also susceptible to exotic NDV originating from migratory birds that play an important role in the transmission and act as the main reservoir of the virus (Zhu *et al*., 2018).

It was recently discovered that there was a significant difference between viruses circulating in the field and viruses used for vaccines. Although ND vaccines have cross protection properties, in terms of biological characteristics, they are particularly different in inducing an immune response. This plays an important role in the epizootiology and the spread of the disease (Munir *et al*., 2012). In the last 5 years, the dominant type of NDV in Indonesia is genotype VII, while the vaccine used is Lasota which belongs to genotype II. So, there is a mismatch between viruses circulating in the field and viruses used for vaccines (Fernandes *et al*., 2014).

Table 2. Homology of gene encoding HN protein of NDV in Sby-Ck01/13, Blt-Ck02/13, Blt-Ck03/13, Sby-Ck35/16 and Pas-Ck36/16 compared to LaSota vaccine as positive control

| No	Isolate	LaSota/16	Sby-Ck01/13	Blt-Ck02/13	Blt-Ck03/13	Sby-Ck35/16	Pas-Ck36/16
1	LaSota/16	100%					
2	Sby-Ck01/13	94%	100%				
3	Blt-Ck02/13	94%	100%	100%			
4	Blt-Ck03/13	94%	100%	100%	100%		
5	Sby-Ck35/16	94%	100%	99%	100%	100%	
6	Pas-Ck36/16	99%	94%	93%	94%	94%	100%
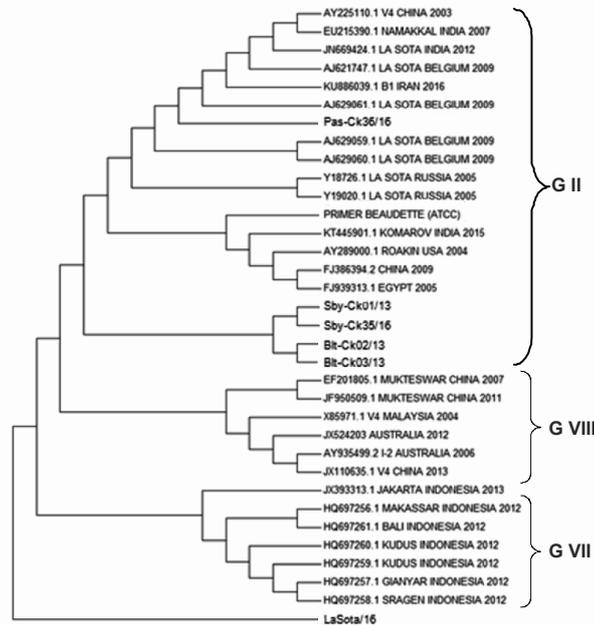
Fig. 2. Phylogenetic tree of partial HN gene nucleotide sequences of Newcastle disease virus (NDV). Beaudette strain as primer resource.

Table 3. Epitope prediction of Sby-Ck01/13

No	Start	End	Peptide	Length	Log score
1	10	23	ENDEREGKAPDIPD	14	19.382
2	45	62	GGQHTRSCRYTDQNFQGR	18	10.429
3	74	84	GCSGDIASGPV	11	9.010
4	93	97	HNYER	5	3.015
5	108	127	CEQQRVGGTYPPRLYRGDRQ	20	16.944
Total			5		

Based on phylogenetic analysis conducted, nucleotide sequences of HN genes might be different from isolate Pas-Ck36/16 and the other isolates, as shown in Fig 2. In this study, one isolate from Pasuruan (Pas-Ck36/16) district was like the original ND viruses in nature because NDV still circulates in environment.

In our study, we predicted the B cell epitopes of the region of HN protein based on the BepiPred method on IEDB. These methods were used to predict specific areas in proteins that bind to the B cell receptor, and must be on the surface and immunogenic. Bepipred linear epitope prediction tool is the program based on a hidden Markov model. The best single method to predict linear epitope and a trend scale has the positive predictions that are characterised by E as B cell linear epi-

Table 4. Epitope prediction of Pas-Ck36/16

No	Start	End	Peptide	Length	Log score
1	10	21	ENDEREGKALDV	12	11.326
2	47	59	TRSCRHTDDFQGR	13	8.701
3	75	83	DIQASGPVS	9	6.885
4	91	95	HNYER	5	3.015
5	109	126	QWVGGTYPPRLYRGDRQ	18	14.152
Total			5		

tope (Erik *et al*., 2006). B cell epitope is accessible and antigenic (Vita *et al*., 2015).

To prevent NDV in Indonesia, a variety of containerised strains in the form of live vaccines and inactive vaccines are used. Live vaccines are mostly formulated from lentogenic strains in the form of attenuated vaccines, but sometimes are also formulated from genotypes II such as B1, C30, and Beudatte. There is also a recombinant vaccine containing the HN and F coding genes of genotype V. That is probably the cause of the changes in the new varian circulating in the field (Afonso & Miller, 2014). Therefore, even though vaccinations have been carried out, they still cannot stop ND outbreaks in Indonesia. Although the differences are likely to be geographically determined, the original viruses circulating in nature are more stable than attenuated vaccines. Further work should be performed is to determine whether the NDV isolate from Surabaya will be used in vaccine development to minimize infected chickens or not.

In conclusion, our experiment showed five lentogenic isolates of NDV: Sby-Ck01/13, Blt-Ck02/13, Blt-Ck03/13, Sby-Ck35/16 and Pas-Ck36/16. Based on homology and phylogenetic analysis, it was shown that one isolate from Pasuruan district (Pas-Ck36/16) was like the original ND viruses in nature.

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