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RESEARCH ARTICLE

Genotype Characterization of Newcastle Disease Virus Isolated from Commercial Chicken Flocks in West Java, Indonesia

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ABSTRACT

Newcastle disease (ND) is a worldwide very contagious poultry disease, caused by Newcastle disease virus (NDV). Despite the vaccination, ND outbreaks in Indonesia's commercial chicken flocks have been reported regularly. Our study aimed to determine the genotype of isolates and genetic relatedness with other Indonesia's NDVs published on the GenBank. Four NDV isolates were obtained from vaccinated flocks in 2011, 2014, 2015 in West Java, Indonesia. Two NDVs belong to virulent strain and the other two belong to avirulent strain. Phylogenetic analyses of F gene revealed that NDV/Ck/BGR/11 and NDV/Ck/GS/14 belong to genotype VII sub-genotype (h) and (i); whilst NDV/Ck/CJR/15 and NDV/Ck/BGR/15 belong to genotype II. The virulent NDVs were clustered in the same genotype and closely related to earlier Indonesia's NDVs isolated in 2007, 2009 and 2010. Result of current study showed that recent NDVs of sub-genotype VIIh and VIIi circulating in commercial chicken farm in West Java, Indonesia have high similarity with NDVs isolated during 2007 and 2010 in Indonesia. Our findings may be valuable for future studies to develop improved control and diagnostic strategies of ND.

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INTRODUCTION

Newcastle disease (ND) is a very contagious infection that affects more than 250 species of birds. The causative agent of ND is Newcastle disease virus (NDV) otherwise known as Avian Paramyxovirus type 1 (APMV–1) (Alexander, 2000). According to genome length and F gene sequence, APMV-1 strains are divided into two major subdivisions: class I and II (Czegledi *et al.,* 2006). Class I NDV is divided into one genotype (Diel *et al.,* 2012; Courtney *et al.,* 2013; Snoeck *et al.,* 2013) which has been recovered from waterfowl and shorebirds which are mostly avirulent to chickens (Czegledi *et al.,* 2006; Miller *et al.,* 2010), whereas class II NDV is mainly obtained from poultry, pet, and wild birds and categorized into eighteen genotypes and some sub-genotypes (Diel *et al.,* 2012; Snoeck *et al.,* 2013; Choi *et al.,* 2014).

The genetic class classification of NDV evolves continuously. Recently, a classification system was introduced by Diel *et al.* (2012) based on mean inter-populational evolutionary distances between previous existing NDV genetic groups. If the cut off value of mean inter-populational evolutionary distance is more than 10% compared to previous existing NDV genetic groups, it will be assigned to new genotype, whereas those whose cut off value ranges between 3 to 10% will be assigned to new sub-genotype (Diel *et al.,* 2012). A vast genetic diversity has been demonstrated within NDV strains based on partial or complete nucleotide sequences phylogenetic analysis of the F gene (Miller *et al.,* 2010).

Intensive vaccination programs have been implemented for all commercial flocks in Indonesia, however ND continues to be a serious problem for the poultry industry (Samal, 2011) due to its genetic variation (Dimitrov *et al.,* 2017). ND outbreaks causing up to 80%

mortality in commercial chicken occurred in Indonesia by 2009 and 2010 (Xiao *et al.,* 2012). Recently, NDV infection of genotype VII has been reported causing outbreaks in several commercial poultry farms in Indonesia (Xiao *et al.,* 2012; Dharmayanti *et al.,* 2014). West Java is one of the Indonesia's provinces that have experienced recurrent ND outbreak because of its high density of poultry population. Understanding NDV genotypes circulating in commercial flocks is needed to control the disease in these area, nevertheless such information is inadequate. Our study aimed to determine the genotype of isolates and genetic relatedness among Indonesia's NDVs published in the GenBank.

MATERIALS AND METHODS

Newcastle disease virus isolates: Four pathotype-characterized isolates used in this research were obtained from the repository of the Immunology Laboratory, Faculty of Veterinary Medicine, Bogor Agricultural University. Two isolates belong to virulent NDV strain (NDV/Ck/BGR/11; NDV/Ck/GS/14) and the other two belong to avirulent NDV strain **(**NDV/Ck//CJR/15; NDV/ Ck/BGR/15) (Putri *et al.,* 2017).

RNA isolation: NDV RNA was extracted from harvested virus in embryonated chicken eggs allantoic fluids using QIAamp@ Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer instruction (Qiagen, 2014). This method used 140µl of sample suspension for extraction. The final volume obtained was 60µl.

Amplification: RT-PCR was performed using Qiagen® One-step RT-PCR kit (Qiagen, Germany) according to manufacturer instruction. Amplification for F gene was set as 50°C for 40 min followed by initial denaturation at 94°C for 2 min and 35 cycles of denaturation at 94°C for 60 s, annealing at 52°C for 60 s, extension at 72°C for 60 s and final extension at 72°C for 10 min (Yuan *et al.,* 2012).

Primers: A set of primer NDV-F-Forward 5'-ATGGGCTCCAAACCTTCTAC-3' and NDV-F-Reverse 5'-TTGTAGTGGCTCTCATC-3' were used to generate 1662 bp amplicon target (Yuan *et al.,* 2012)

Detection of PCR products: PCR products were separated by electrophoresis in 1.5% agarose gel in 1x Tris acetate EDTA (TAE) buffer and stained with ethidium bromide, compared with 1 Kb molecular mass ladder and visualized by ultraviolet (UV) transillumination.

Sequence alignment: Purified PCR products were sequenced by First Base Company (Malaysia) with the primers (NDV-F-Forward and NDV-F-Reverse) based on a variable portion (nt 1–1662) covering the complete F gene. The positive results of PCR products were sequenced using BigDye® Terminator v3.1 cycle sequencing Kit (Thermo Fisher Scientific, USA) according to manufacturer instruction. The obtained sequence was edited using BioEdit Sequence Alignment Editor Version 7.0. The subsequent phylogenetic analysis was implemented using MEGA version 6 (Tamura *et al.,* 2013).

Phylogenetic analyses and genotype classification criteria: The phylogenetic tree was constructed by Neighbor-Joining Kimura 2 parameter model with 1000 bootstrapped replications. Genotype and sub-genotypes nomenclature were assigned based on Diel *et al*'s classification (Diel *et al.,* 2012; Miller *et al.,* 2015). The mean evolutionary distance between genotypes was determined using the maximum composite likehood model (Tamura *et al.,* 2004). The phylogenetic tree was constructed to predict the genetic relatedness and phylogenetic distribution of the viruses using nucleotide sequences data of 58 reference strains obtained from the GenBank database, representing all NDV genotypes and earlier sequences reported from Indonesia.

RESULTS

Reverse transcription polymerase chain reaction: RT-PCR reaction was utilized to achieve molecular characterization analysis. A set of primer (NDV-F-Forward 5′-ATGGGCTCCAAACCTTCT-3′ and NDV-F-Reverse 5′-TGTAGTGGCTCTCATC-3′) were used to generate 1662 bp amplicon target of the NDV F gene (Yuan *et al.,* 2012). Amplified PCR products examination by electrophoresis on 1.5% agarose gel resulted in the expected sizes of amplicons for all NDV isolates (Fig. 1).

Sequencing and sequence alignment: Four viruses, two virulent strains (NDV/Ck/BGR/11 and NDV/Ck/GS/14) and two avirulent strains (NDV/Ck/CJR/15 and NDV/Ck/ BGR/15) were selected for this study. These NDVs were sequenced based on F gene which covers 1662 nucleotides. F gene sequencing by using NDV-F-Forward and NDV-F-Reverse primers revealed in readable sequences. To obtain the exact alignment result, all sequences were cut into the same length in 1569 nucleotides (nucleotide number 40 to 1607). Based on the nucleotide sequence alignment, all sequence isolates were identical in 1224 nucleotides (78.01%) but different from each other in 345 nucleotides (21.99%).

Comparison of F gene nucleotide sequences demonstrated that NDVCk/BGR/11 has 91.44, 84.10 and 84.16% nucleotides sequence similarity with NDV/Ck/GS/14, NDV/Ck/CJR/15 and NDV/Ck/BGR/15 respectively. NDV/Ck/GS/14 has 84.23 and 84.29% nucleotides sequence similarity with NDV/Ck/CJR/15 and NDV/Ck/BGR/15 respectively. The nucleotide sequence of NDV/Ck/CJR/15 and NDV/Ck/BGR/15 were 99.94% similar. F gene nucleotide sequences comparison between the virulent NDVs and the earlier Indonesia ND isolates (GenBank database) showed NDV/Ck/BGR/11 has 92.08–99.23% nucleotides sequence similarity with earlier Indonesia's NDVs, and the virus is closely related to NDV/Ck/Banjarmasin-010/10, NDV/Ck/Gianyar-013/10, NDV/Ck/Sragen-014/10, NDV/Ck/Kudus-017/10 and NDV/Ck/Kudus-018/10. The NDV/Ck/GS/14 showed 92.15–97.89% homologous nucleotide sequence with earlier Indonesia's NDVs isolated in 2007, 2009 and 2010. It is also closely related to NDV/Ck/Makasar-003/10, NDV/Ck/Sukerejo-019/10 and NDV/Ck/Bali-020/10. The homologous nucleotide sequence between studied viruses and earlier Indonesia isolates is presented in Table 1.

Table 1: The homologous nucleotide sequence and evolutionary distance between studied isolates and earlier Indonesia's ND isolates

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13
1) NDV/Ck/Bali-1/07[a]		0.0524	0.0568	0.0728	0.0728	0.0728	0.0728	0.0530	0.0734	0.0785	0.0619	0.1558	0.1552
2) NDV/Ck/Makassar-003/10[a]	94.76		0.0096	0.0683	0.0683	0.0683	0.0683	0.0057	0.0690	0.0760	0.0211	0.1539	0.1533
3) NDV/Ck/Bali-020/10[a]	94.32	99.04		0.0715	0.0715	0.0715	0.0715	0.0089	0.0722	0.0792	0.0243	0.1552	0.1558
4) NDV/Ck/Kudus-018/10[a]	92.72	93.17	92.85		0.0000	0.0000	0.0000	0.0677	0.0006	0.0083	0.0779	0.1564	0.1558
5) NDV/Ck/Kudus-017/10[a]	92.72	93.17	92.85	100.00		0.0000	0.0000	0.0677	0.0006	0.0083	0.0779	0.1564	0.1558
6) NDV/Ck/Sragen-014/10[a]	92.72	93.17	92.85	100.00	100.00		0.0000	0.0677	0.0006	0.0298	0.0779	0.1564	0.1558
7) NDV/Ck/Gianyar-013/10[a]	92.72	93.17	92.85	100.00	100.00	100.00		0.0677	0.0006	0.0298	0.0779	0.1564	0.1558
8) NDV/Ck/Sukorejo-019/10[a]	94.70	99.43	99.11	93.23	93.23	93.23	93.23		0.0683	0.0754	0.0204	0.1552	0.1545
9) NDV/Ck/Banjarmasin-010/10[a]	92.66	93.10	92.78	99.94	99.94	99.94	99.94	93.17		0.0077	0.0785	0.1571	0.1564
10) NDV/Ck/Bogor/11[b]	92.15	92.40	92.08	99.17	99.17	97.02	97.02	92.46	99.23		0.0856	0.1590	0.1584
11) NDV/Ck/GnSindur/14[b]	93.81	97.89	97.57	92.21	92.21	92.21	92.21	97.96	92.15	91.44		0.1577	0.1571
12) NDV/Ck/Cianjur/15[b]	84.42	84.61	84.48	84.36	84.36	84.36	84.36	84.48	84.29	84.10	84.23		0.0006
13) NDV/Ck/Bogor/15[b]	84.48	84.67	84.42	84.42	84.42	84.42	84.42	84.55	84.36	84.16	84.29	99.94	

Abbreviation: NDV, Newcastle disease virus; Ck, Chicken; [a]All earlier Indonesia's ND isolates (GenBank database); [b]All studied isolates were collected from vaccinated chickens; The percentage of homologous nucleotide sequence among studied isolates are shown in bold; The highest percentage of homology between isolates with earlier isolates shown in grey shading.

Table 2: Amino acid substitution in the hypervariable region and neutralizing epitopes of F gene sequences of the isolates

Isolates	Hypervariable Region													Neutralizing Epitopes							
	4	10	11	13	20	21	27	52	63	78	93	101	121	72	74	75	78	79	157-169	170	171	343
Consensus[c]	K	P	A	L	M	L	C	I	V	K	T	R	V	D	E	A	K	A	SIAATNEAVHEVT	D	G	L
1) NDV/Ck/Bali-1/07[a]	N	-	I	P	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	N	-	-
2) NDV/Ck/Makassar-003/09[a]	-	-	T	P	-	-	-	-	-	R	-	K	I	-	-	-	R	-	-	N	-	-
3) NDV/Ck/Bali-020/10[a]	-	-	T	P	I	-	-	-	-	R	-	K	I	-	-	-	R	-	-	N	-	-
4) NDV/Ck/Kudus-018/10[a]	-	-	V	-	-	-	-	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-
5) NDV/Ck/Kudus-017/10[a]	-	-	V	-	-	-	-	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-
6) NDV/Ck/Sragen-014/10[a]	-	-	V	-	-	-	-	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-
7) NDV/Ck/Gianyar-013/10[a]	-	-	V	-	-	-	-	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-
8) NDV/Ck/Sukorejo-019/10[a]	-	-	T	P	-	-	-	-	-	R	-	K	I	-	-	-	R	-	-	N	-	-
9) NDV/Ck/Banjarmasin-010/10[a]	-	-	V	-	-	-	-	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-
10) NDV/Ck/Bogor/11[b]	n.a.	n.a.	n.a.	n.a.	-	-	-	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-
11) NDV/Ck/GnSindur/14[b]	n.a.	n.a.	n.a.	n.a.	I	-	-	-	-	R	-	K	I	-	-	-	R	-	-	N	-	-
12) NDV/Ck/Cianjur/15[b]	n.a.	n.a.	n.a.	n.a.	A	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-
13) NDV/Ck/Bogor/15[b]	n.a.	n.a.	n.a.	n.a.	A	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-

Abbreviation: NDV, Newcastle disease virus; Ck, Chicken; [a]All earlier Indonesia's ND isolates (GenBank database); [b]All studied isolates were collected from vaccinated chickens; [c]The consensus amino acid sequence (Umali *et al.,* 2013).

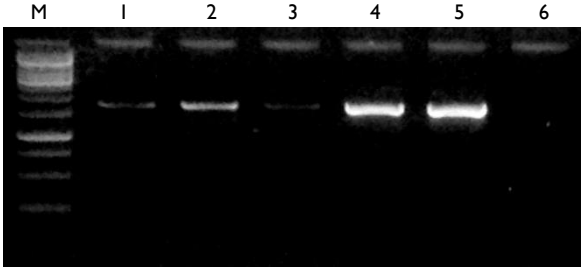

Fig. 1: Fusion gene amplification results. RT-PCR amplification of the Newcastle disease virus F gene which gave a product size of 1662 bp. The amplicons were electrophoresed in 1.5% agarose gel. Lanes: M, molecular size marker; Lane 1, NDV/Ck/BGR/11; Lane 2, NDV/Ck/GS/14; Lane 3, NDV/Ck/CJR/15; Lane 4, NDV/Ck/BGR/15; Lane 5, NDV/Sato (used as positive control) and Lane 6, Non template control.

Phylogenetic analyses and genotype classification criteria: A total 1569 nucleotides encoding 523 amino acid were identified in the F gene of all NDV isolates. Five of six potential N-glycosylation sites were at position 85–87; 191–193; 366–368; 447–449; and 471–473, however N-glycosylation site position 541–543 is not covered in this study. Eleven conserved cysteine residues in location; 76, 199, 338, 347, 362, 370, 394, 399, 401, 424, 523 have been identified in all isolates, except cysteine residue in position 25 which is not detected in NDV/Ck/GS/14. Based on the N-glycosylation site and conserved cysteine residue, there is no change in amino acid sequence of all isolates. Hypervariable region analysis of F gene showed amino acid substitution in five mutation points in the F gene of all isolates. Amino acid substitution K78R and D170N also were detected within two F gene neutralizing epitope of NDV/Ck/GS/14 isolate (Table 2).

The phylogenetic analyses of F gene revealed that NDV/Ck/BGR/11 and NDV/Ck/GS/14 belong to genotype VII (Fig. 2). The criterion to separate genotypes is the cut off distance value being more than 10% (0.1) (Tamura *et al.,* 2004). The distance between these groups is 0.156 while the distance within sub-genotypes is under 10%, so these isolates were clustered in two different sub-genotypes. NDV/Ck/BGR/11 belongs to sub-genotype (i) and was grouped along with NDV/Ck/Banjarmasin-010/10, NDV/Ck/Gianyar-013/10, NDV/Ck/Sragen-014/10, NDV/Ck/Kudus-017/10 and NDV/Ck/Kudus-018/10 while NDV/Ck/GS/014 isolate belongs to sub-genotype (h) and was grouped along with NDV/Ck/Makasar-003/10, NDV/Ck/Sukerejo-019/10 and NDV/Ck/Bali-020/10. The two other avirulent NDV isolates belong to genotype II and were grouped along with Lasota vaccine.

DISCUSSION

Genotype characterization of NDV strains were mostly accomplished by the F gene as it codes a number of functionally important amino acid structures (de-Leeuw *et al.,* 2003). Analysis of potential N-glycosylation site and cysteine residue showed conserved amino acid sequence in all isolates and amino acid substitution is detected in hypervariable region and neutralizing epitope. It is unclear whether mutation is a part of NDV adaptive mechanism in evading immune response of vaccinated chicken or if it is actually an effect of selective immune pressure exerted on viral particle as a vaccination consequence (Umali *et al.,* 2013).

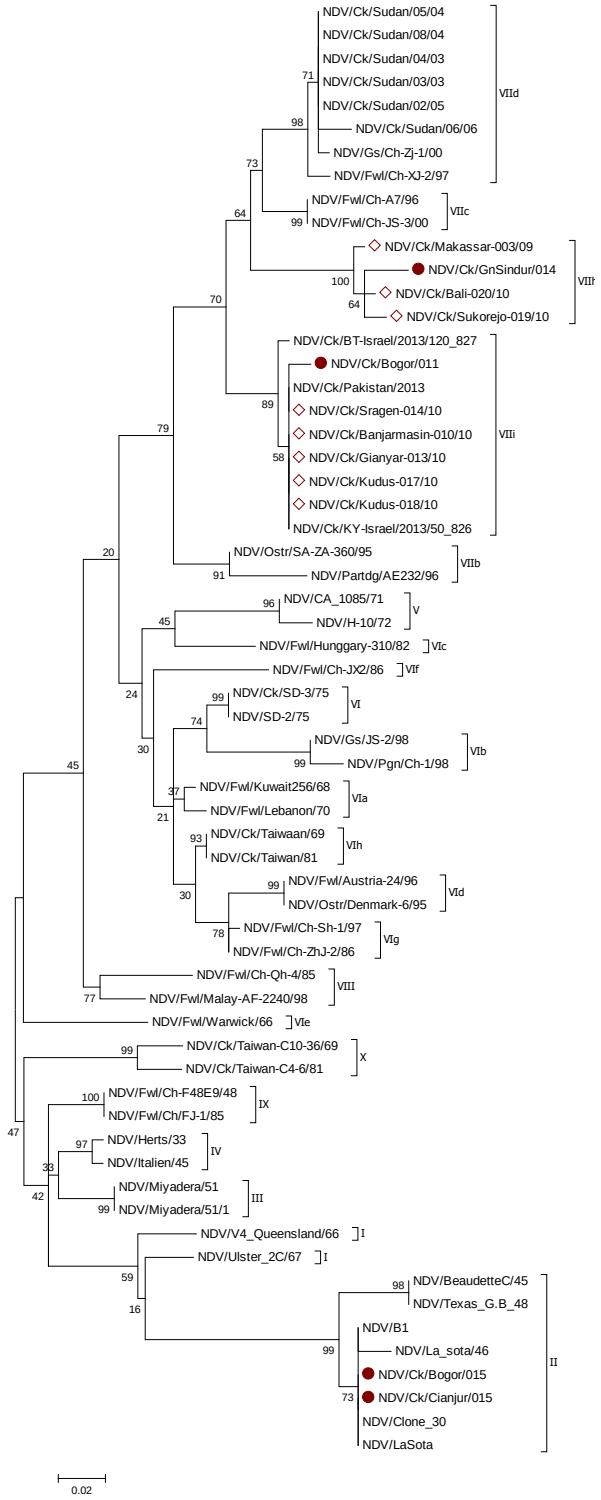

Fig. 2: Phylogenetic tree of partial Newcastle disease viruses F (fusion) gene, (◇) All earlier Indonesia's ND isolates (GenBank database); (●) All studied isolates. The region of tF gene from 40–1607 was analysed by MEGA version 6.

Phylogenetic topology and evolutionary distances between different taxonomic groups were used to assign genotypes and sub-genotypes according to previously established criteria (Diel *et al.*, 2012). A vast amount of sequence data on NDVs isolated around the world has been documented over the years and available for sequence comparison and phylogenetic analysis to estimate the genotype and identify the origin of NDV outbreaks. In this study, the genotype of four isolates, two virulent strains (NDV/Ck/BGR/11 and NDV/Ck/GS/14) and two avirulent strains (NDV/Ck/CJR/15 and NDV/Ck/BGR/15), were characterized, and nucleotide sequences data of 58 reference strains obtained from the GenBank database (Table 2) were used to cluster these isolates based on genotype and sub-genotype. Phylogenetic analyses of the F gene revealed that NDV/Ck/Bogor/11 and NDV/Ck/GS/14 belong to genotype VII sub-genotype (h) and (i), while NDV/Ck/CJR/15 and NDV/Ck/BGR/15 belong to genotype II.

Since the first Indonesia's ND outbreak reported in 1926, ND spreads and becomes endemic in all Indonesia's province. Until now we cannot find free ND area in Indonesia. Two NDV genotypes, VII and VIII, were reported as novel viruses in South Africa, Asia, and several European countries in 1990s (Abolnik *et al.*, 2004; Liu *et al.*, 2007). To date, genotype VII of NDVs is the causative agent responsible for the fourth panzootic, which is predominant and continued circulating in the domestic poultry of Asia, Africa, and Europe (Wang *et al.*, 2013; Miller and Koch, 2013; Yang *et al.*, 2017). Shohaimimi *et al.* (2015) also stated that between 2000 to 2010, genotype VII has caused outbreaks in Malaysia.

Genotype VII became more prevalent in this region, which is further divided into eight sub-genotypes (VIIa–VIIh). The sub-genotype VIIh which circulated between 2009-2012 in Indonesia, Cambodia, and China is most closely related to the Indonesia/Bali/01/2007 strain (Adi *et al.*, 2010). The new sub-genotype VIIi is closely related to earlier isolates from Indonesia and with isolates collected in Pakistan and Israel. The NDV sub-genotype VIIi has been responsible for ND outbreaks in Pakistan from 2012 (Miller *et al.*, 2015). Nucleotide sequence among the isolates and comparison between earlier NDV isolates indicate that the NDV strains circulating in Indonesia between 2011-2015 have close relationship with NDV strains causing outbreak in Indonesia's poultry in 2007 and 2010.

The avirulent NDV strain isolates, NDV/Ck/CJR/15 and NDV/Ck/BGR/15, were closely related with live Lasota vaccine in genotype II. The detection of vaccine-like strains mostly due to the use of live vaccines (Snoeck *et al.*, 2009) and indicated that the vaccines used in this farm curb disease but cannot prevent viral shedding (Kapczynski and King, 2005). Intensive vaccination programs, often improperly implemented in developing countries, may contribute to the evolution of avirulent viruses into their virulent counterpart (Kapczynski *et al.*, 2013). Our study revealed that virulent NDV strains were circulating in vaccinated chicken flocks in West Java, Indonesia. ND outbreaks among vaccinated flocks suggest that vaccination strategies are not effective in controlling the virus yet (Nakamura *et al.*, 2014) so we need to revise the NDV control strategy.

Conclusions: Result of current study showed that NDV new sub-genotype VIIh and VIIi which predominantly circulating in commercial chicken farm in West Java, Indonesia have high similarity with Indonesia's ND viruses isolated in 2007 and 2010. Findings of vaccine like strain and outbreaks in vaccinated flocks also indicated that vaccination program implemented have not been effective yet. Our findings may be valuable for

future studies to develop improved control and diagnostic strategies for the disease.

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Authors contribution: DDP executed the work (collection of data, analysis and writing of manuscript); EH, RDS and AS participated in designing the study and drafting of the manuscript; NLPIM participated in analysis and interpretation of data and drafting of the manuscript; ONP participated in analysis and interpretation of data and writing of manuscript. All authors read and approved the final manuscript.

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