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Molecular Characterization and Pathogenesis of Newcastle Disease Virus Isolated from Brontok Eagle in West Java, Indonesia

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ABSTRACT

Newcastle disease (ND) is a contagious disease in poultry and numerous birds of various ages. Eagle is considered a potential reservoir for ND transmission as a wild bird. This research was conducted to molecularly characterize Newcastle disease virus (NDV) isolated from ND cases in Brontok Eagle and analyze the pathogenesis in chicken embryos. qRT-PCR was conducted as confirmation of NDV without mixing Avian Influenza (AI). Sequencing the fusion (F) and haemagglutinin-neuraminidase (HN) genes from the three NDVs was performed with a specific primer. Amino acid sequence compared with other NDV from Genbank. Pathogenicity, genetic variation, distance, and phylogenetic studies were analyzed using bioinformatics software (MEGA-X). This study analyzed pathogenesis based on lesions and distribution of viral antigens in chicken embryos infected with NDV. Observations were based on tissue lesions with HE and IHC staining. NDV isolated from three Brontok Eagles is classified as velogenic strain, virulent NDV (KRQKRF), and belonging to Genotype VII subgenotype VII.2. The NDV was detected in various organ lesions, more severe in the pulmo, trachea, proventriculus, and intestine of chicken embryos. That is still similar to the previous case reports in the field. These results show that NDV, which infected Brontok Eagle, has similar molecular characteristics and pathogenesis in chickens. These cases could be a threat to the poultry industry. Further research, surveillance, and monitoring of wild birds are needed to obtain more NDV epidemiological information in wild birds.

1. Introduction

Newcastle disease (ND) is a contagious disease in poultry that cause economic losses to the poultry industry. ND is caused by viruses from the family *Paramyxoviridae*, genus *Avulavirus*, species *Avian paramyxovirus* serogroup *Avian paramyxovirus* Type I (APMV-I). Viral replication occurs in the cytoplasm. APMV-I is a synonym for NDV, with various genotypes (Alexander and Senne 2008). NDV were classified into two classes, I and II, and were further grouped into at least 21 genotypes with many sub-genotypes based on a complete coding sequence analysis of the fusion gene (F-gene) (Dimitrov *et al.* 2019). Class I NDV isolates was classified into one genotype (genotype 1) containing three sub-genotypes. In contrast, class II viruses were divided into 15 genotypes (I to XV) and several sub-genotypes (Diel *et al.* 2012). This classification system was widely adopted and identified three more genotypes (XVI, XVII, and XVIII) (Courtney *et al.* 2013; Dimitrov *et al.* 2016; Snoeck *et al.* 2013). Development of a dichotomous naming system to designate sub-genotypes introduced to trace viral ancestry, and this naming system produces three New Genotypes in Class II (XIX, XX, and XXI) (Dimitrov *et al.* 2019).

The pathotype of ND is classified into viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic, and asymptomatic. The pathogenicity of the NDV can be analyzed both biologically and molecularly. Biologically, it can be done by analyzing MDT, ICPI, and IVPI. The molecular pathogenicity and genotyping can be determined by characterizing the

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F gene in the cleavage site area (Alexander 1989; OIE 2012). Interaction among HN and F proteins plays a role in infection and viral virulence (Stone-Huslander and Morisson 1997). The HN protein, together with F, supports the meeting and fusion between infected cells and surrounding cells. HN also has six glycosylation sites which play an important role in protein folding, stability, maturation of antigenicity, and determinants of virus virulence classification. (McGinnes and Morrison 1995; McGinnes *et al.* 2002).

APMV-I can infect various domestic birds such as chickens, turkeys, geese, ducks, pigeons, ostriches, cockatoos, and parrots (Alexander 1989; Chang *et al.* 2001; Ellakany *et al.* 2019; Kommers *et al.* 2002). Wild birds such as eagles have a broader range to fly, so they have the potential to spread disease more widely. The cruising range can expand along with changing seasons (Widiana 2017). NDV circulating in poultry farms in Indonesia is genotype VII (Xiao *et al.* 2012; Goraichuk *et al.* 2020; Wibowo *et al.* 2017). NDV reported in East Java, Indonesia, in ducks caused by NDV genotypes II, VI, and VII (Putri *et al.* 2021), in geese (Putri *et al.* 2019) are NDV lentogenic which has a cleavage pattern similar to that of La Sota genotype II. Pigeons with symptoms of torticollis have also been reported. Based on histopathological examination and serological identification by agar gel precipitation test indicated that it was caused by NDV infection (Dewandaru *et al.* 2020). Brontok eagles that show clinical symptoms of Newcastle disease have been reported in West Java (Anonim 2019), but research on the virus has not been carried out. This study was conducted to characterize the molecular characteristics of NDV isolated from Brontok eagles and its pathogenesis in chicken embryos.

2. Materials and Methods

2.1. Ethical Clearance

This research received ethical approval from the Ethics Commission of Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta (EC. No:00014/EC-FKH/Int./2021).

2.2. Study Period and Location

This research was conducted from March-August 2021 at Microbiology Laboratory Gadjah Mada University Yogyakarta and Disease Investigation Center Lampung, Indonesia.

2.3. Samples

Specimens from the ND case on three Brontok eagles from the Eagle Conservation Center in Garut, West Java. One isolate from dead Eagle and two isolates from sick Eagle showed clinical symptoms leading to Newcastle Disease, such as weakness, anorexia, and diarrhea. NDV isolates have been isolated at the Subang Disease Research Center, West Java, Indonesia. Further research on virus characterization has never been done before.

2.4. RNA Extraction and q-RT_PCR of Isolates

RNA extraction used Purelink Viral RNA/DNA mini kit. The procedure used is following the manufacturer's instructions (Invitrogen). qRT-PCR to confirm that Isolate was NDV without mixture with AI. Primers which qRT-PCR AI used 1 set primer-probe M gene AI, forward-5' AGATGAGYCTTCTAACCGAGGTCG 3', Reverse-5'-TGCAAANACATCYTCAAGTCTCTG-3', Probe-5'FAM-TCAGGCCCCCTCAAAGCCGA-TAMRA 3' (BBVet Wates) and 1 set primer-probe M gene ND, Forward-ACAACCAAGTGAGGTGAGTACTTG, Reverse-5'-GACTCCCTTTCTCTGATTGTCCAT-3' and probe-5'FAM-CGTTTCCAGTCGTTGGATTTAC-TAMRA 3'(AAHL, Geelong).

The mix reagent for deoxyribonucleic acid (DNA) amplification follows the manufacturer's instructions (Ambion AgPath-IDTM). The mix solution was amplified using the ABI-7500 Real-Time PCR Thermo Cycler machine. The result is a curve and ct values. The value of Ct <40 is positive, 40-45 is indeterminate, and Ct>45 is negative.

2.5. Amplification and Sequencing of F and HN Gene

The results of the isolates examination showed positive ND and negative AI followed by Reverse Transcriptase Polymerase Chains Reaction (RT-PCR). Each amplification process was carried out by RT-PCR using forward and reverse primers to amplify the nucleotide sequences of the F and HN genes. The F sets of primers consisting of 4 pairs of overlapping primers with the product along 1662 base pair consist of F1 (forward 5'-AGAGTGTGGATCCCAACCAG-3' and reverse 5'-GTGGATACAGACCCTTGAATCTTG-3'), F2(forward5'-GCAGGGATTGTAGTAACAGGAGAT-3' and reverse 5'-CCAAGAGTTGAGTCTGTGAGTCAT-3'), F3 (forward 5'-ACTACAGTGTTCGGGCCACA-3' and reverse 5'-AGCCTCAGAGTTATCCCGTCTAAT-3'), F4 (forward 5'-GTTTGAGCGGCAACACATC-3' and

reverse 5'-GTTCTACCCGTGTATTGCTCTTTG-3'). HN sets primer consisting 3 pairs of overlapping primers amplifying 1666 nucleotide consist of HN1 (forward 5'-CCCACAACATCCGTTCTACC-3' and reverse 5'-CGAAGCACACCAAGTGCTAA-3'), HN2 (forward 5'-TTAGCACTTGGTGTGCTTCG-3' and reverse 5'-ACCGTGAGAATTCTGCCTTC-3'), HN3 (forward 5'-TGAGGACCCAATGCTGACTA-3' and reverse 5'-CCCCGAATAGGGTATTGGAT-3'). The DNA amplification reagent is superscript III platinum Taq-HiFi. Each pair of primers conducted by procedure follow the manufacturer's instructions (Invitrogen). Amplification by using a Thermocycler engine. The PCR product was continued sanger sequencing sent to 1st BASE (Malaysia).

2.6. Phylogeny and Genetic Analysis

MEGA-X software is used in sequence data assembly and sequence alignment. Linking the sequences resulting from the alignment of each primer into a single unit. The complete F and HN gene sequences of available class II NDV isolates were downloaded from GenBank of the National Center for Biotechnology Information. The pathogenicity of the isolates was analyzed based on the cleavage site of the F gene. Genetic variations were observed in the nucleotide base sequences F and HN genes. The datasets were used to estimate the pairwise and the mean interpopulational distances (Kumar *et al.* 2018). Phylogenetic analyses were conducted using the Neighbor-Joining method (Tamura *et al.* 2004).

2.7. Re-Isolation Newcastle Disease Isolates in Embryonic Chicken Eggs (ECE)

The isolates and negative control of 0.2 ml were propagated in 10-day-old embryonated chicken eggs. The eggs that had been inoculated were put into Incubator 37°C for five days. Embryo death was observed every 24 hours. The Allantoic fluid of dead chicken embryos was harvested to be tested by hemagglutination (HA) and hemagglutination inhibition (HI).

2.8. Collecting the Embryo, Hematoxylin, Eosin (HE), and Immunohistochemistry (IHC)

The pathologic changes in the dead embryo were observed. The tissues of embryos collected and fixed by 10% NBF were processed and embedded in paraffin. Duplicate sections were stained with HE and IHC. The IHC reagent includes primary rabbit anti-NDV polyclonal antibody in 1:500 dilution (Dako, S3022), secondary antibody Dako REAL™ envision™/HRP, rabbit/mouse (K5007), Dako REAL™ DAB + chromogen in Dako REAL™ substrate Buffer (K5007) for IHC. Staining procedure according to manufacturer's instructions.

2.9. Histopathology Analysis

Histopathologic lesions were observed of necrosis, hemorrhagic, and inflammatory cell infiltration, focal (low severity), multifocal (moderate severity), and visible diffuse (high severity). The distribution of the NDV was observed in various tissues. Analysis of immunopositive in tissue with a scoring system of cells showed positive reactions to immunohistochemical staining in each field of view. The scoring adapted from Etriwati *et al.* (2017) consists of low (1-10), medium (11-20), and high (>20) immunopositive cells against NDV. Data were analyzed descriptively.

3. Results

3.1. qRT-PCR

Molecular NDV identification using the qRT-PCR method based on the amplification of the M gene for NDV screening. Three virus isolates from DIC Subang confirmed NDV without mixing with AI. The identification results showed that samples E013, E005, and E004 were positive for NDV with ct values of 15.71, 17.41, and 17.93, and all were negative for AI.

3.2. Amplification and Sequencing of F and HN Gene

The results are ABI files assembled and analyzed using MEGA-X. The assembly results of F and HN gene sequences (n = 6) from the three NDVs (E005, E013, and E004) obtained in this study were submitted to GenBank and are available with accession numbers MW811474, MW811475, MW811476, MW811477, MW811478, and MW811479.

3.3. Phylogeny and Genetic Analysis

Phylogenetic analysis of the F genes showed that the isolates in this study were closely related to the previous Indonesian NDV isolate, namely subgenotype VII.2. (Figure 1). All isolates of this study contained the aminoacid sequences KRQKRF at positions 112-117 at the cleavage site. The genetic distance of the

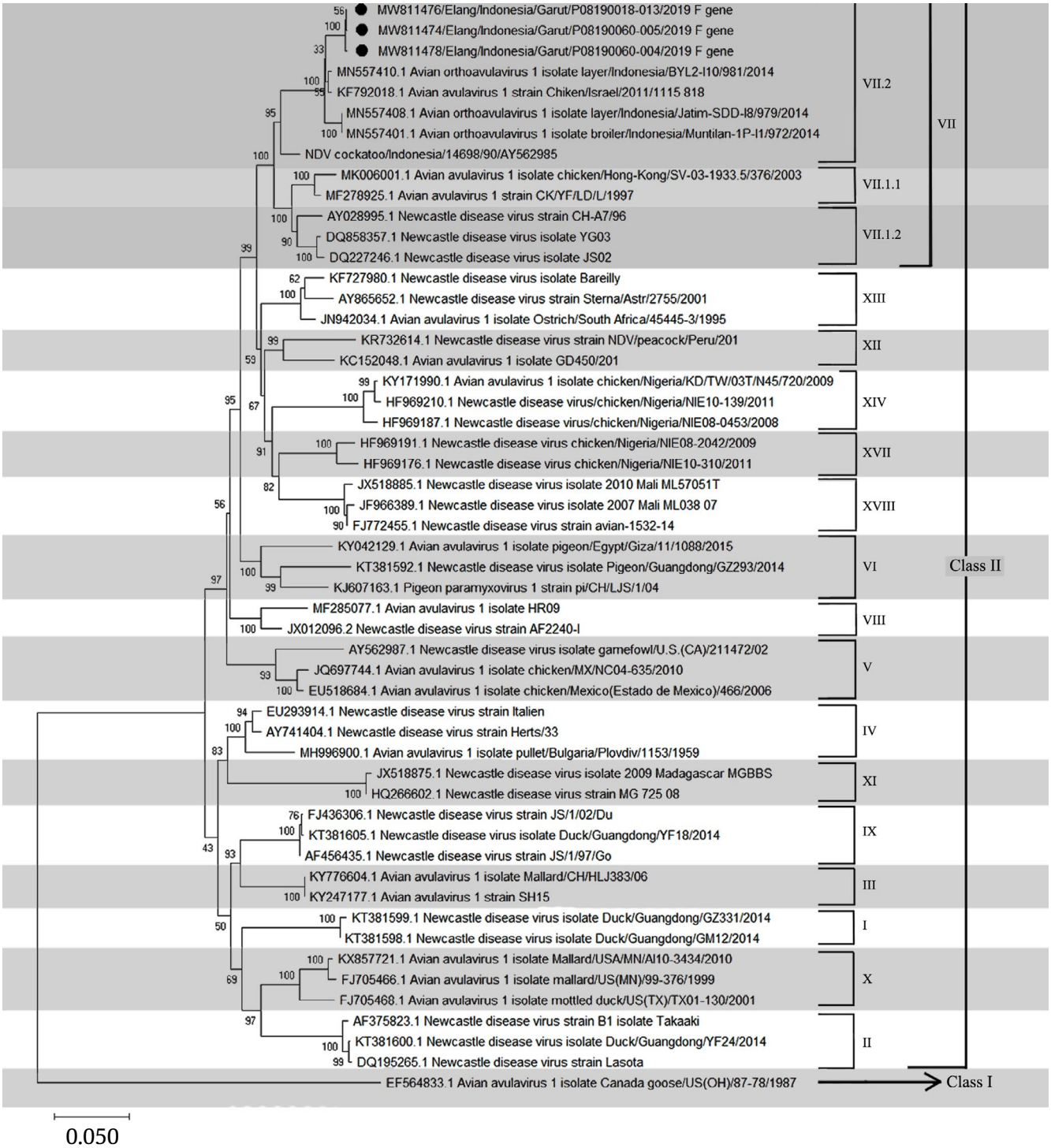

Figure 1. Phylogenetic analysis is based on the full-length nucleotide sequence (n = 1662) of the fusion gene of selected isolates representing class II NDV subgenotypes (n = 52) and class I as outgroup (n = 1). Tree construction was created by the Neighbor-Joining method using MEGA-X software. The isolates in this study were marked with black dot (●)

Table 1. Genetic distances based on the F gene between isolates in this study and NDV isolates from various genotypes from the data in the GenBank

Newcastle Disease Virus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
MN557410.1_layer/ Indonesia/ BYL2-I10/981/2014 (VII.2)																										
MN557408.1_layer/ Indonesia/Jatim-SDD-I8/979/2014 (VII.2)	0.018																									
• MW811478/Elang/ Indonesia/Garut/ P08190060-004/2019 (VII.2)	0.020	0.026																								
• MW811476/Elang/ Indonesia/Garut/ P08190018-013/2019 (VII.2)	0.022	0.027	0.002																							
• MW811474/Elang/ Indonesia/Garut/ P08190060-005/2019 (VII.2)	0.024	0.029	0.004	0.002																						
KR732614.1_NDV/peacock/ Peru/201 (XII)	0.118	0.128	0.133	0.130	0.130																					
JX518885.1_2010_Mali_ ML57051T (XVIII)	0.115	0.124	0.123	0.122	0.122	0.124																				
HF969191.1_chicken/ Nigeria/NIE08-2042/2009 (XVII)	0.117	0.124	0.133	0.131	0.131	0.123	0.097																			
KY171990.1_chicken/ Nigeria/KD/TW/03T/ N45/720/2009 (XIV)	0.136	0.137	0.144	0.141	0.141	0.133	0.127	0.120																		
KF727980.1_Bareilly (XIII)	0.088	0.097	0.103	0.103	0.103	0.105	0.103	0.102	0.124																	
JX518875.1_2009_ Madagascar_MGBBS (XI)	0.196	0.195	0.205	0.205	0.204	0.215	0.204	0.200	0.234	0.187																
KX857721.1_Mallard/USA/ MN/AI10-3434/2010 (X)	0.165	0.168	0.177	0.177	0.177	0.189	0.180	0.180	0.200	0.171	0.184															
MF285077.1_isolate_HR09 (VIII)	0.118	0.123	0.129	0.127	0.127	0.136	0.130	0.140	0.146	0.122	0.176	0.155														
KF792018.1_Chiken/ Israel/2011/1115_818 (VII.2)	0.005	0.013	0.015	0.018	0.020	0.119	0.115	0.119	0.136	0.090	0.195	0.166	0.118													
DQ858357.1_isolate_YG03 (VII.1.2)	0.071	0.079	0.085	0.083	0.083	0.112	0.111	0.115	0.127	0.089	0.195	0.162	0.113	0.071												
FJ436306.1_strain_JS/1/02/ Du (IX)	0.149	0.153	0.164	0.162	0.164	0.174	0.161	0.153	0.192	0.142	0.150	0.114	0.128	0.150	0.136											
EF564833.1_ Canada_goose/ US(OH)/87-78/1987 (*class I)	0.439	0.437	0.431	0.433	0.435	0.421	0.429	0.417	0.435	0.413	0.439	0.386	0.399	0.435	0.415	0.417										

Table 1. Continued

Newcastle Disease Virus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
MK006001.1_chicken/Hong-Kong/SV-03-1933.5/376/2003 (VII.1.1)	0.082	0.093	0.095	0.093	0.094	0.114	0.119	0.125	0.132	0.096	0.207	0.175	0.129	0.083	0.053	0.152	0.433									
KY042129.1_pigeon/Egypt/Giza/11/1088/2015 (VI)	0.120	0.121	0.130	0.128	0.129	0.136	0.133	0.145	0.153	0.119	0.203	0.189	0.113	0.121	0.110	0.153	0.428	0.119								
JQ697744.1_chicken/MX/NC04-635/2010 (V)	0.134	0.140	0.145	0.144	0.144	0.139	0.136	0.128	0.147	0.127	0.178	0.162	0.111	0.135	0.118	0.131	0.416	0.127	0.132							
KT381605.1_Duck/Guangdong/YF18/2014 (IX)	0.150	0.153	0.164	0.163	0.165	0.176	0.160	0.156	0.195	0.144	0.151	0.115	0.128	0.150	0.137	0.003	0.420	0.153	0.154	0.132						
MH996900.1_pullet/Bulgaria/Plovdiv/1153/1959 ((IV)	0.143	0.147	0.154	0.152	0.154	0.160	0.151	0.153	0.176	0.140	0.144	0.137	0.121	0.143	0.131	0.103	0.408	0.145	0.157	0.134	0.104					
KY776604.1_Mallard/CH/HLJ383/06 (III)	0.144	0.149	0.153	0.151	0.153	0.175	0.160	0.165	0.198	0.152	0.164	0.125	0.130	0.144	0.140	0.085	0.397	0.150	0.162	0.139	0.085	0.106				
DQ195265.1_strain_Lasota (II)	0.182	0.188	0.198	0.198	0.198	0.215	0.191	0.189	0.223	0.181	0.197	0.111	0.159	0.183	0.166	0.120	0.413	0.190	0.190	0.168	0.121	0.142	0.127			
AF375823.1_strain_B1_isolate_Takaaki (II)	0.177	0.184	0.193	0.193	0.193	0.212	0.186	0.189	0.221	0.181	0.195	0.108	0.157	0.178	0.164	0.117	0.409	0.184	0.184	0.169	0.118	0.144	0.125	0.010		
KT381599.1_Duck/Guangdong/GZ331/2014 (I)	0.178	0.179	0.187	0.188	0.188	0.196	0.184	0.171	0.206	0.179	0.187	0.131	0.167	0.175	0.172	0.128	0.403	0.178	0.189	0.173	0.127	0.151	0.129	0.143	0.138	

nucleotide sequences between the isolate samples in this study is shown in Table 1.

3.4. Re-Isolation Newcastle Disease Isolates in Embryonic Chicken Eggs (ECE)

Embryonic chicken eggs were infected by three viruses isolated from the Brontok eagle and one as a control. Except for negative controls, all embryos infected with isolates died less than 60 hours after infection. Three NDV samples were analyzed with HA and HI tests. The results showed that the titer of viral hemagglutination ranged from 1:128 to 1:256, which could be inhibited by standard NDV-positive serum. Negative HA-HI for control.

3.5. Collecting the Embryo, Histopathology, and Immunohistochemistry (IHC)

The dead embryos were hemorrhagic, had no feathers, and were stunted (Figure 2). Hemorrhages in almost all visceral organs. No gross lesions were observed in the control embryos of ECE. Histopathologic lesions in the organ are summarized in Table 2. Viral antigen was demonstrated in the many organs of the embryo.

4. Discussion

The isolate of this study was NDV without a mixture with AI. The real-time PCR method targeting the M gene only be used to identify the presence of RNA from APMV-1. It cannot be used in differentiation between velogenic, mesogenic, and lentogenic strains (Cattoli *et al.* 2011).

NDV genotype VII is circulating in commercial chickens and other birds in Indonesia and many countries in Asia. NDV reported in Banjarmasin, Sragen, Giyanyar (Xiao *et al.* 2012) and subgenotype VII.2 in Boyolali, Kulon progo and Muntilan (Goraichuk *et al.* 2020). The presence of wild birds can be a mechanical intermediary for NDV transmission to commercial chickens. Wild birds may contact the environment contaminated with NDV from chickens infected with ND (Garcia *et al.* 2013). This result is the first reported NDV subgenotype VII.2 in Brontok Eagle in Indonesia. Similar cases have also been reported in wild birds in Turkey (Turan *et al.* 2020).

The fusion of NDV has 12 conserved cysteine residues in position 25, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424, and 523 (Qin *et al.* 2008). The NDV in this study had cysteine residues conserved on amino acids except for Y^{25}. The isolate from the Brontok eagle had a residue that was conserved in almost all of these locations except A^{79} to V^{79} based

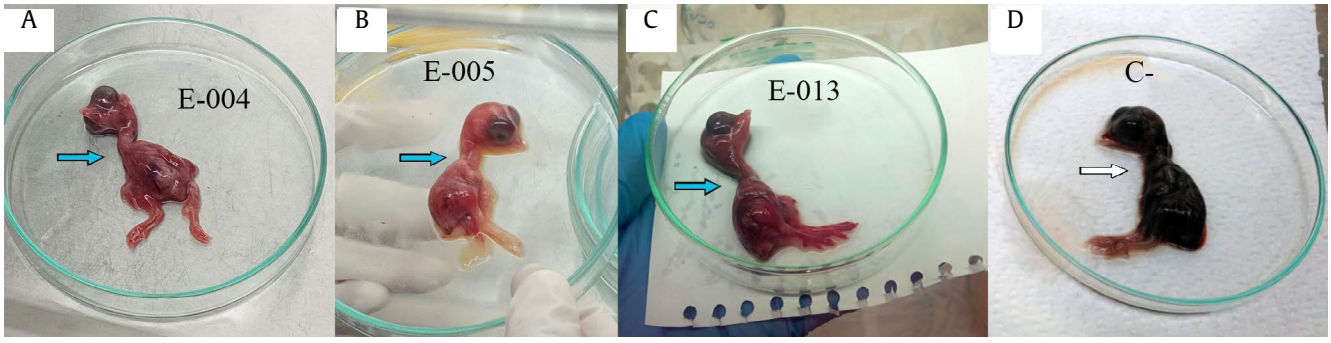

Figure 2. Gross lesions of embryos after infection with NDV isolated from eagles. (A) ECE infected with NDV E-004, blue arrow, (B) ECE infected with NDV E-005, blue arrow, (C) ECE infected with NDV E-013, blue arrow and (D) ECE as negative control, white arrow

Table 2. Distribution and intensity of histological lesion and IHC staining for NDV

Sample	Staining	Pulmo	Liver	Heart	Kidney	Proventriculus	Brain	Intestinum	Skin
E013	HE(Haemorraghes)	+++	+++	++	++	+++	++	++	++
	HE(necrosis)	+++	+++	++	++	+++	++	+++	++
	HE(Infiltration of imflamatory cell)	+++	+++	++	++	+++	++	++	++
	IHC	+++	+++	+++	++	+++	++	++	++
E005	HE(Haemorraghes)	+++	+++	+++	++	++	++	++	++
	HE(necrosis)	+++	+++	+++	++	++	++	++	++
	HE(Infiltration of imflamatory cell)	+++	++	+++	++	+++	++	++	++
	IHC	+++	++	+++	++	+++	++	++	++
E004	HE(Haemorraghes)	+++	+++	+++	++	+++	+	++	++
	HE(necrosis)	+++	+++	+++	++	+++	++	++	++
	HE(Infiltration of imflamatory cell)	+++	+++	+++	++	+++	+	++	++
	IHC	+++	+++	+++	++	+++	+	++	++

Histopathologic lesions in HE staining +: low severity, ++: moderate severity, +++: high severity. Immunopositive reaction of IHC staining +: low, 1-10, ++: medium, 11-20, +++: high, > 20 immunopositive cells against NDV

on seven neutralization epitopes located at the 72, 74, 75, 78, 79, 151-171, and 343 (Munir *et al.* 2012). Indonesian NDV virus isolated from cockatoo in 1990 (AY562985), layer from Boyolali (MN557410.1), and goose in East Java 2019 (Putri *et al.* 2019) have an arrangement on the cleavage site with the pattern 112RRQKR↓F117. The NDV in this study with a black dot mark has a pattern of 112KRQKR↓F117; there is a substitution R112 to K112 similar to NDV that was previously reported in Indonesia (Angeliya *et al.* 2015; Wibowo *et al.* 2017; Xiao *et al.* 2012). According to OIE (2012), strains with amino acid sequence patterns at the cleavage site that have at least three basic amino acids are included in velogenic strains. These samples are different from the ND Lasota as a vaccine strain used on the poultry farm in Indonesia. The Lasota strain, an amino acid sequence at the cleavage site ^{112}GRQGR↓L^{117}, is a lentogenic NDV.

Three regions in the HN protein responsible for hemagglutination activity: ^{234}NRKSCSV^{240}, ^{314}FPVYGGL^{320}, and ^{399}GAEGRIL^{405}, are not modified in NDV from Eagle. Neutralization epitope at position ^{193}LSGCRDHSH^{201}, ^{263}K, ^{287}D, ^{321}K, ^{332}GK^{333}, ^{345}LDEQDYQIR^{353}, ^{356}K, ^{481}N, ^{494}D, ^{513}RVTRVSSSS^{521}, found at NDV of the Eagle and they are not modified, similar to previous Indonesian NDV. All isolates showed two mutations at G494D and I514V compared to the Lasota vaccine strain (Li *et al.* 2020; Ren *et al.* 2016). Mutation in the neutralizing epitopes of the HN proteins of NDVs may produce antigenic variants and escape antibody neutralization (Qin *et al.* 2008; Snoeck *et al.* 2013; Zhu *et al.* 2016). The HN gene analysis of NDV from Brontok eagle has conserved 13 cysteine residues. Cysteine plays a role in stabilizing the structure of the HN protein (McGinnes and Morrison 1995; Ren *et al.* 2016). Role of N-linked carbohydrates in HN glycoproteins for cell attachment. The HN protein of this isolate contained six potential N-glycosylation sites N-X-S/T at 119, 341, 433, 481, and 508, which were conserved but at 508 not conserved (D-I-S) and specifically for samples coded E004 at 119 (N-G-G). The glycosylation site of the HN gene plays an important role in affinity for receptors on target cells so that it is involved in influencing the virulence of NDV (Panda *et al.* 2004).

The genetic distance in the F gene between the three isolates from Brontok Eagle was 0.02-0.04% (Table 1), and the homology value was 99.6-99.86%. The genetic distance with the previous Indonesian isolates was 2.0-2.9%, with a 97.1-98.0% homology value. This value is almost the same as the results reported by Xiao *et al.* (2012) in isolates from Indonesia, including genotype VII, which had a homology value between 96%-100%. NDV in this study has varied genetic distances with NDV from different hosts. The genetic distance between Peacocks and Pigeons is 12.8-13.3%, duck is 16.3-18.8%, and mallard is 15.3-17.7%. Based on this analysis, all NDVs in this study still have a genetic distance closer to the previously reported NDV originating from commercial chickens in Indonesia than NDV from wild bird hosts from other countries. Wild birds can also be infected with ND from the contamination of the NDV circulating in commercial chickens (Xiang *et al.* 2017).

The distribution of lesions and viruses in chicken embryos was carried out to see the pathogenesis of NDV in chicken embryo tissue (Table 2). The dose used for infection in ECE was 0.2 ml with a viral content of 7log2 or 2^7 HAU per ECE. The infective dose of ECEs is viral titer 23, while the infective dose is at the chicken is viral titer 2^6 (Rue *et al.* 2011). There was no significant difference in the timing of embryo death and lesions gross NDV infection with a viral dose of 2^6-2^{10} (Qosimah *et al.* 2018). The embryos died in 48 hours and showed clinical signs such as hemorrhage, no feather, and stunted (Figure 2).

Pulmo as respiratory organs were observed in every dead chicken embryo. The presence of the virus in respiratory epithelial cells can be seen from the immunopositive reaction on IHC staining (Figure 3). The virus will encourage the immune system by infiltrating the virus's inflammatory cells and phagocytes. The response is the presence of hemorrhage and necrosis caused by the viral invasion. Immunopositive reaction in pulmo (Al-garib *et al.* 2003) and trachea of chicken embryo (Wijaya *et al.* 2018).

The characteristic lesion of the brain due to NDV infection is perivascular cuffing (Figure 3). Hemorrhage and necrosis are also seen in the brain

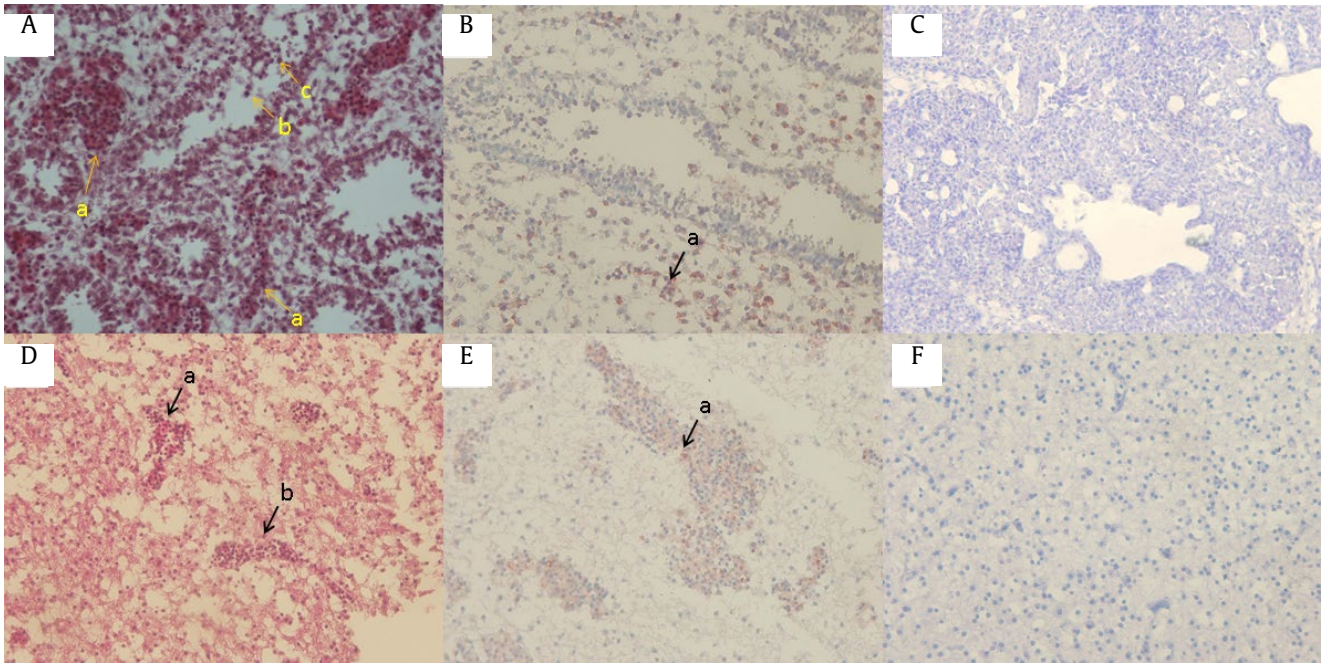

Figure 3. Histopathological lesion in HE and IHC staining Histopathology changes and distribution of NDV in internal organs of chicken embryos post infected with NDV from eagles. (A) Pulmo microscopic observation showed hemorrhages (a), mononuclear cell infiltration (b), epithelial necrosis (c). (B) Pulmo immunopositive reaction was distributed on cytoplasm of epithelial cell (a). (C) Pulmo in normal condition as negative control. (D) Brain microscopic observation showed haemorrhages (a), perivascular cuffing (b). (E) Brain immunopositive reaction was distributed to glial cell and mononuclear cells of perivascular cuffing (a). (F) Brain in normal condition as negative control. HE staining (A and D), IHC staining (B, C, E, and F)

after NDV infection. The characteristic lesions in the embryonic brain after NDV infection are congestion, hemorrhage, and inflammation (Wijaya *et al.* 2018). Inflammation begins with spreading macrophages in the perivascular cuffing then spreads to astrocytes and microglia in the brains of chickens infected with NDV (Kommers *et al.* 2002) and immunopositive reactions to NDV can be found in inflammatory cells and astroglia (Etriwati *et al.* 2017) in chicken embryo brain (Wijaya *et al.* 2018).

The most extensive and intense viral antigen staining was observed on the epithelial of the respiratory and gastrointestinal tracts of chicken embryo. The crucial part of this study was reported NDV velogenic subgenotype VII.2, which is very close to the circulating NDV in poultry. This proves that the Eagle can be a carrier of NDV transmission. These results require particular attention to the risk of spreading ND between wild and poultry.

Conflict of Interest

There is no conflict of interest regarding the publication of this article.

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