Jurnal Kedokteran Hewan March 2019, 13(1):10-14
P-ISSN : 1978-225X; E-ISSN : 2502-5600 DOI: https://doi.org/10.21157/j.ked.hewan.v13i1.5832

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS CIRCULATES IN SOME DISTRICTS IN ACEH

M Daud AK[1]*, Surachmi Setiyaningsih[2], and Idwan Sudirman[3]
[1]Microbiology Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia
[2]Virology Laboratory, Faculty of Veterinary Medicine, Bogor Agricultural Institute, Bogor, Indonesia
[3]Virology Laboratory, Faculty of Veterinary Medicine, Bogor Agricultural Institute, Bogor, Indonesia
*Corresponding author: m.daud.ak@unsyiah.ac.id

ABSTRACT

The objectives of this study were to assess the diversity of Newcastle Disease Virus (NDV) isolates; to detect and isolate NDV from poultry; and to identify and characterize NDV by serological and molecular assays. A total of 84 cloacal-oropharynx isolates of poultry was collected from privately owned poultries and poultry markets from 12 districts in Aceh Besar and Banda Aceh. Screening was performed by real time reverse transcriptation-polymerase chain reaction (rRT-PCR) to 15 isolates of poultry. Selected isolates were inoculated in 9-11 days old embryonated specific pathogen free (SPF) eggs and showed positive hemagglutination (HA). Characterization was performed through hemagglutination inhibition (HI) test using Komarov and Hitchner B1 antisera, elution test, RT-PCR and realtime RT-PCR fusion (F). All isolates had a higher affinity to Komarov antisera (titer up to 4 log), indicating virulent strain. This was supported by elution test which showed that 93.66% isolates were virulent and 6 % non-virulent. In conclusion, RT-PCR can detect Matrix gene from 15 isolates (100%), while Fusion gene only detected from 11 isolates (73.3%). rRT-PCR is more capable of detecting antigenic diversity compared to RT-PCR.

Key words: antigenic diversity, Newcastle Disease, RT-PCR, rRT-PCR, virulence

ABSTRAK

Tujuan penelitian ini adalah mengetahui keragaman isolat virus Newcastle Disease, mendeteksi dan mengisolasi virus pada ayam buras dan unggas air, dan mengidentifikasi dan mengkarakterisasi virus secara serologi dan molekuler. Sebanyak 84 sampel isolat kloaka dan orofaring dari ayam buras, unggas air, dan ayam kampung yang berasal dari pekarangan dan pasar unggas dari 12 wilayah di kabupaten Aceh Besar dan kota Banda Aceh digunakan dalam penelitian ini. Uji skrining dilakukan dengan real time reverse transcriptation-polymerase chain reaction (RT-PCR) terhadap 15 sampel isolat dari ayam buras dan unggas air. Beberapa isolat di inokulasikan pada telur ayam embrio specific pathogen free (SPF) umur 9-11 hari dan semua isolat menunjukkan titer hemagglutination (HA) positif. Karakterisasi dilakukan dengan uji hemagglutination inhibition (HI) menggunakan serum Komarov dan B1, uji elusi, RT-PCR, dan rRT-PCR gen fusion. Semua isolat mempunyai afinitas yang tinggi terhadap serum Komarov dengan titer di atas 4 log yang mengarah kepada strain virulent. Uji elusi menunjukkan sebesar 93,66% isolat virulent dan hanya 6% isolat non-virulent. RT-PCR dapat mendeteksi gen matrix dari 15 isolat (100%), sementara gen fusi hanya terdeteksi pada 11 isolat (73,3%). rRT-PCR lebih mampu mendeteksi keragaman dibandingkan dengan RT-PCR.

Kata kunci: keragaman antigenic, Newcastle Disease, RT-PCR, rRT-PCR, virulensi

INTRODUCTION

Newcastle disease (ND) is a viral disease caused by *Paramyxovirus* tipe A, *Avulavirus* spp. *Mononegaverales.* The virus is classified as ssRNA virus, with a total of 16.000 nucleotides in the genome and replicates inside host cytoplasm. ND virus can be inactivated by heating at 56° C for 3 hours and 60° C for 30 minutes, inside acidic pH, and is also sensitive to ether, formaline, and phenol. However, ND virus can remain viable in a damp temperature, especially inside fowl feces (Aldous and Alexander, 2001).

ND is a contagious and fatal viral disease that attacks all fowls. Clinical manifestation varies depending on virus strain, species, fowl age, secondary disease and body immunity. According to the clinical manifestation, ND is classified into 5 main groups: velogenic visetropic, velogenic neurotropic, mesogenic, lentogenic and asymptomatic (OIE, 2012). In Indonesia, ND is widely spread inside and outside Java. Currently, all regions in Indonesia are endemic for ND and there is no ND-free region (Tarmudji, 2005). ND attacks usually increase at the start of rainy season and peaks at mid season or during the transition of rainy season to dry season.

ND virus genome contains six Open Reading Frames which codes nucleocapsid protein, several phosphoprotein (P), matrix protein (M), fusion protein (F), three core proteins and three envelop proteins, including two large glycoproteins hemagglutinin-neuraminidase (HN) and large RNA-directed RNA polymerase (L). In addition there are non-structural protein (V) and second protein (W) that are produced during transcription of P gene into RNA (Oberdorfer and Werner, 1998). Out of the six proteins, only two play a role in immune process, F and HN proteins, which can stimulate production of protective antibody (Cho *et al.*, 2008).

Fusion protein (precursor F glycoprotein) plays an important role during host cell infection because this protein can split into trypsin-like enzyme which aids fusion between virus and host cell membrane, promoting virus entry into host cell (Rout, 2007). Hemagglutinin-neuraminidase (HN) is an immunoprotective glycoprotein (immunogenic determinant) which acts as surface antigen on ND virus envelope. The M, F, and HN protein are located on the virus envelope. F and HN aid virus entry and exit from host cell while M plays a role in morphogenesis and budding of ND virus. NP protein mediates encapcidation of RNA genome to form

nucleocapsid that becomes the template for viral transcription and replication. P protein is important for viral RNA synthesis and form a separate complex with NP and L proteins and nucleocapsid (EFSA, 2007).

Generally, ND virus attacks fowls such as ducks, geese, doves, parakeet and several other fowls. ND virus has been isolated from about 236 fowl species. Chickens are the most vulnerable while waterfowls are the most resistant to infection. Generally, virus isolated from waterfowls is weakly pathogenic towards domestic fowls. Several low virulence isolates from waterfowls that migrate in United States are used as B1 strain and La Sota commercial vaccines (Lomniczi *et al.*, 1998).

Virus virulence is determined by several genetic factors. Virus virulence also involves tissue or organ tropism which is related to host immune system. Virulence determination can also be performed molecularly (molecular pathotyping) by examining the amino acid sequence at cleavage site of F protein. Lentogenic viruses contain monobasic ^{112}G/E-K/R-Q-G/E-R^{116} amino acid motif and L (leucine) on residue 117, while mesogenic and velogenic strains contain multibasic ^{112}R/K-R-Q/K/R-K/R-R^{116} amino acid motif and F (phenylalanine*)* on residue 117. Protein F with monobasic motif is split by extracellular trypsin-like protease found on respiratory and digestive tracts, while those with polybasic motif is split by intracellular furin like protease enzymes that is found in various host tissues, resulting in fatal systemic reaction (OIE, 2012).

ND virus classification into genotypes is based on nucleotide sequence of fusion genes which is classified into class I and II. Class I virus is divided into 9 genotypes, which are often found in domestic fowls and waterfowls and are less virulent. Class II virus is divided into 16 genotypes, often found among chickens, pet birds and waterfowls. Genotypes I, II, III, IV and IX have a short genome, 15,186 nucleotides long, and are called "early" virus (found between year 1930-1960). Genotypes V, VI, VII, VIII and X are 15.192 nucleotides long and are grouped as "late" virus (found after year 1960). All class II viruses are virulent except genotype I which caused outbreak in Australia from 1998 to 2000, and used as live vaccines. Genotype II from class II is also non-virulent and hence widely used as vaccine such as La sota, B1 and VG/GA (Hines and Miller, 2012; Miller *et al.*, 2013).

Aceh Livestock Office reported that outbreaks of ND still occurred from 2011 to 2012; there were nearly 100 cases in each sub-district. Case investigation is still conventional in the form of clinical manifestation examination, anatomic pathology changes and serologic testing. Virus isolation has not been performed and misdiagnosis with Avian Influenza (AI) commonly occured.

Previous study reported antigenic and genetic diversity among NDV isolated from chickens in Aceh (Darniati, 2014). Therefore, investigators saw a need to conduct further study to confirm and document the presence of diversity as well as adding isolates from other fowls. This study's general objective was to investigate the diversity in NDV that circulates in Aceh. Specific objectives of this study include detection and isolation of NDV from broiler chicken and waterfowls, identification and characterization of virus based on their physical, serological and molecular profiles.

MATERIALS AND METHODS

Study Sample

Cloaca and oropharynx swab sampels were pooled based on swab type, fowl type and sampling date. Pooling was conducted on 5-7 individuals with individual sample volume of 100 µL.

Viral RNA Isolation

RNA isolation was conducted using High Pure Viral Nucleic Acid Kit (Roche®) following standard protocol provided by the manufacturer.

Real-Time Reverse-Transcriptation Polymerase Chain Reaction (rRT-PCR) Test

The application used was Ag_Path ID TM One-Step RT-PCR kit from Ambion®. The primers used were matrix forward (M+4100): 5′-AGTGATGTGCTC GGACCTTC-3′ and matrix reverse (M-4220) 5′-CCTGAGGAGAGGCATTTGCTA-3′. The probe used was M+4169 5′-FAM TTCTCTAGCAGTGGGAC AGCCTGC-3′. Result was analyzed by Applied Biosystems 7500 Real-Time PCR System software (CVL, 2007)

Virus Isolation from Embryonated Chicken Egg

Virus isolation was performed from individuals with positive cloaca and oropharynx samples for gene matrix with rRT-PCR. Cloaca and oropharynx swab samples as inoculum were suspended with Penicillin-streptomycin (200.000 IU), and 0.2 ml were inoculated into Spesific Pathogen Free (SPF) embryonated chicken eggs aged 9-11 days through allantoic cavity. Inoculated eggs were incubated and observed for 4-7 days at 37° C with humidity 60-65% (OIE, 2012).

Hemagglutination (HA) and Hemaglutination Inhibition (HI) Test

HA and HI tests for isolated virus was performed according to procedure suggested by OIE (2012).

Real-time RT-PCR test with Oligonucleotide (F) Primers

Testing on fusion (F) gene was done using oligonucleotide primer for forward primer Fusion F+ 4829 5′-GGTGAGTCTATCCGGARGATACAAG-3′ and matrix reverse M+ (M-4894) 5′-AGCTGTTGCAACCCAAG-3′. The probe used was M+ (4939) 5′- FAMAAGCGTTTCTGTCTCCTTCCTCCA [BHQ-3]′ (CVL, 2007).

RT-PCR test with Oligonucleotide (M) Primers

Gene M was amplified using primer 4100F-5090R (Kim *et al.*, 2007).

RESULTS AND DISCUSSION

Identification and Isolation of ND Virus using real time RT-PCR

Detection and isolation on 15 cloaca and oropharynx samples from non-broiler chickens, broiler chickens, layer chickens and waterfowls (ducks and geese) from 2 locations, which were Public Community Facility (PCF) and Public Slaughter House (PSH) from 4 sub-districts in Banda Aceh and Aceh Besar using matrix primer showed that 10 samples were positive while 5 samples were negative. Positive and negative samples were spread in 2 locations from 4 sub-districts. Swab samples were taken from fowls that showed clinical and subclinical symptoms. Sample data and rRT-PCR amplification can be seen in Table 1.

The result of realtime RT-PCR amplification showed positive result as exponential phase line that cuts threshold line and is located at a different position on plateau phase. 10 samples that were positive for matrix gene from real time RT-PCR test also showed positive result for HA test. Hence, real time RT-PCR can detect all samples from various locations in Banda Aceh and Aceh Besar. This is influenced by high sensitivity of rRT-PCR to detect live or dead virus. Generally, positive sample showed Ct (Cycle Threshold) value between 25 and 37. The smaller the Ct value, the higher the virus concentration. In waterfowls with subclinical symptoms, virus concentration remained high because viruses were still excreted even without clinical symptom (Kapczynski *et al.*, 2013). Saepulloh and Darminto (2005) reported that waterfowls infected with mesogenic and velogenic NDV were often subclinical but still capable of transmitting to other fowls (carrier). Embryonated chicken egg inoculation results also showed that almost all sample isolates did not cause embryonic death in <90 hours, but had high HA titer of 2^7, positive for fusion gene on RT-PCR and indicated virulent NDV based on elution test.

ND Virus identification with conventional RT-PCR

All samples from non-broiler chickens, broiler chicken, layer chicken and waterfowls were amplified

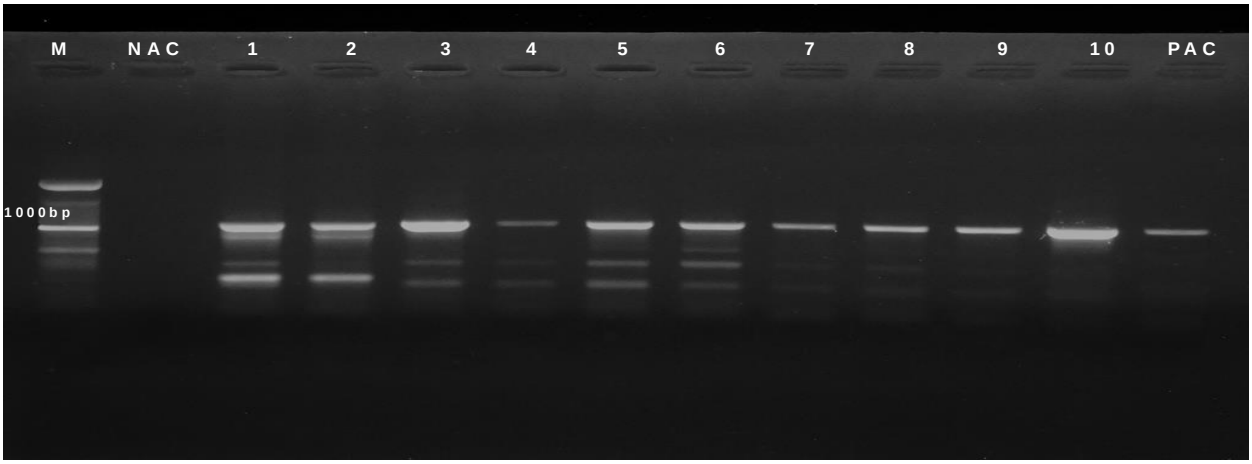

Figure 1. Results of electrophoresis amplification using 991 bp of RT-PCR pruduct (100 bp marker)

Table 1. Results of sample isolation and detection by using real time RT-PCR (Matrix gene)

District	Sub-district	Origin of poultry	Location	Poultry	Ct value
Banda Aceh	Kuta Alam	Kuta Malaka	PSH	Broiler	12.0
	Kuta Alam	Kuta Malaka	PSH	Broiler	18.95
	Kuta Alam	Kuta Malaka	PSH	Broiler	Negative
	Kuta Alam	Lhoksemawe	PCF	Arab chicken	Negative
	Kuta Alam	Lhoksemawe	PCF	Arab chicken	19.0
	Kuta Alam	Lhoksemawe	PCF	Arab chicken	16.80
	Kuta Alam	Lhoksemawe	PCF	Arab chicken	17.38
	Kuta Alam	Lhoksemawe	PCF	Arab chicken	Negative
	Kuta Alam	Lhoksemawe	PCF	Arab chicken	19.0
	Ulee Kareng	Lhok Nga	PCF	Duck	37.42
	Ulee Kareng	Lhok Nga	PCF	Duck	Negative
Aceh Besar	Montasik	Montasik	Farm	Layer	16.41
	Suka Makmur	Suka Makmur	PCF	Geese	Negative
	Suka Makmur	Suka Makmur	PCF	Geese	17.08
	Suka Makmur	Suka Makmur	PCF	Geese	17.38

PSH= Public Slaughter House, PCF= Public Community Facility

using 991 bp 4100F-5090R primer. 8 µL of RNA (the same volume used in realtime PCR) in 25 µl reaction mix One-Step RT-PCR (Roche and Invitrogen). PCR amplification product was visualized in 1% agarose gel and 1xTAE which contained ethidium bromide. PCR products showed thin band were reamplified using Superscripts III (Invitrogen) in a different PCR reaction condition (Roche). Reamplification was performed to confirm whether the band that appeared was contamination or low RNA concentration (Figure 1).

ND Virus F Gene Fragment Detection with rRT-PCR

Fragment F detection with real time RT-PCR from 15 isolates showed 11 isolates positive for F gene (73.33%) while 4 isolates were negative. This occurred because of incompatibility between primer oligonucleotide or probe with ND virus cDNA template (Cattoli *et al.*, 2011). Wise *et al.* (2004) stated that F gene probe for rRT-PCR could detect 10^2-10^4 copy of RNA and at least 10 ND virus particles, hence the concentration of virus that is needed is lower. In addition, rRT-PCR test is more specific for certain antigens (Kencana *et al.*, 2012). Testing using F gene was aimed to detect virulent ND virus strain and differentiated between virus from vaccinated fowl and wild virus. Meanwhile, M gene was used for unvaccinated fowls such as waterfowls (Kim *et al.*, 2006). However, M and F genes were not always detected for all ND virus strains due to frequent mutation on those genes, hence new sequence of M and F genes are needed for rRT-PCR test (Dharmayanti *et al.*, 2014). Specificity and sensitivity for F gene can be increased by reducing annealing temperature or modification of primer and probe (Kim *et al.*, 2008).

Antigenic Diversity Identification with Hemagglutination Inhibition (HI) Test

Optimal antibody titer will be formed if inhibition of agglutination reaction occurs using homologous serum-specific polyclonal ND virus (Miller *et al.*, 2013). Figure 4 shows difference in affinity between serum B1 and Komarov towards virus isolate with variation in antibody titer, where serum B1 showed lower antibody titer (4 Log2 - 6 Log2) compared to Komarov serum that reached 5 Log2 - 9 Log2. Isolate is denoted positive for ND virus when it has reached 4 Log2 - 5 Log2 or with value of 16-32 (OIE, 2012). According to Sa'idu and Abdu (2008), vaccination failure is often caused by difference between vaccine strain and wild virus, hence the vaccine could not protect chickens from VND infection. The higher the antibody titer in a chicken, the more protected they are toward VND infection (Figure 2).

Detection of ND Virus Distribution in Aceh

ND virus is highly prevalent in all regions in Aceh in all fowl species and is classified as velogenic strain both

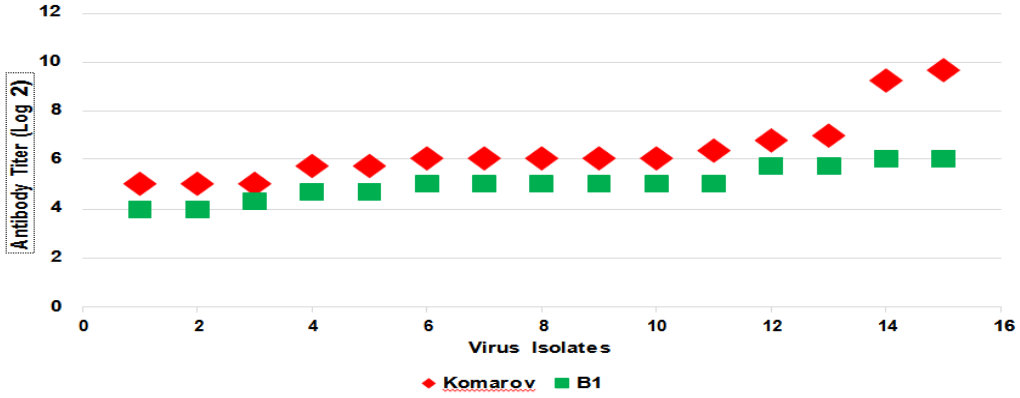

Figure 2. Variations in the affinity of antibody titers (4 Logs and 5 Logs) between two serums (B1 and Komarov) using Hemagglutinin Inhibition test

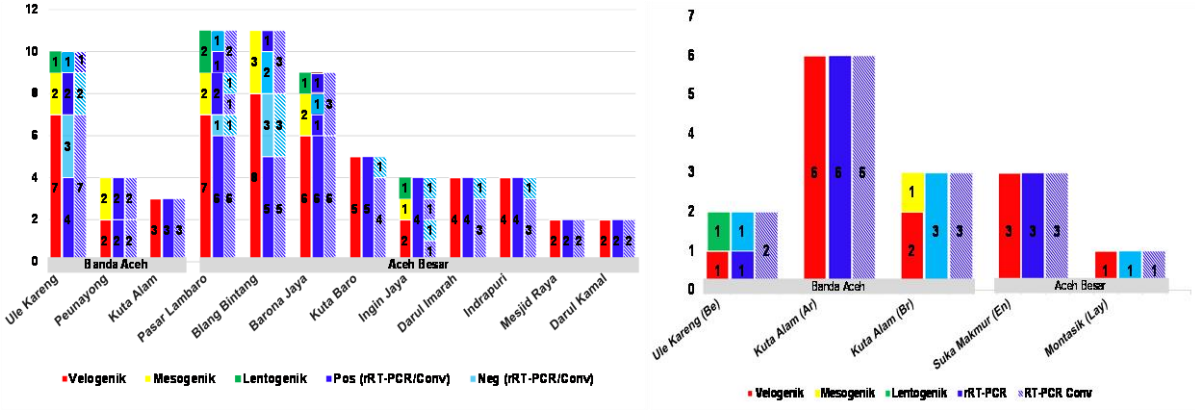

Figure 3. VND distribution in poultry collected from several areas in Aceh Besar and Banda Aceh

antigenically and genetically (Figure 3). The high prevalence of NDV was caused by the absence of proper biosecurity system during maintenance, marketing and management of dead fowls. Waterfowls were often left uncaged and kept in the same cage with native chickens. Inappropriate and aseptic managements of dead fowls is a risk factor for both direct and indirect transmissions, resulting in the appearance of virus diversity due to the difference between vaccine strain and wild strain (Miller *et al.*, 2013).

CONCLUSION

Conventional RT-PCR could detect 100% virulent virus while real time PCR detected 73.33%, hence rRT-PCR is more capable of detecting diversity. Komarov serum had a higher affinity (93.33%) compared to serum B1.

REFERENCES

Aldous, E.W. and D.J. Alexander. 2001. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). **Avian Pathol**. 30(2):117-128.

Cattoli, G., L. Susta, C. Terregino, and C. Brown. 2011. Newcastle disease: A review of field recognition and current methods of laboratory detection. **J. Vet. Diagn. Invest.** 23(4):637-656.

Cho, S.H., H.J. Kwon, T.E. Kim, J.H. Kim, H.S. Yoo, and S.J. Kim. 2008. Variation of a newcastle disease virus hemagglutinin-neuraminidase linear epitope. **J. Clin. Microbiol**. 46(4):1541-1544.

CVL. 2007. Standard operating procedure for real time polymerase chain reaction detection of virulent Newcastle disease virus in clinical specimens. Tanzania, Central Veterinary Laboratory**.**

Darniati. 2014. Deteksi Molekuler dan Keragaman Virus Newcastle Disease pada Ayam Kampung di Wilayah Aceh. **Thesis**. Palce Published: Institut Pertanian Bogor, Bogor.

Dharmayanti, N.L.P.I., R. Hartawan, D.A. Hewajuli, and R. Indriani. 2014. Phylogenetic analysis of genotype VII of new castle disease virus in Indonesia. **Afr. J. Microbiol. Res**. 8(13):1368-1374.

EFSA. 2007. "Regarding a request from the European commission to review Newcastle disease focusing on vaccination wordwide in order to determine its optimal use for disease control purpose." **The EFSA Journal, European Food Safety Authority**. 477**:**1-24.

Hines, N.L. and C.L. Miller. 2012. "Avian paramyxovirus serotype-1: A review of disease distribution, clinical symptoms, and laboratory diagnostics." **Vet. Med. Int.** 2012:708216.

Kapczynski, D.R., C.L. Afonso, and P.J. Miller. 2013. Immune responses of poultry to Newcastle disease virus. **Dev. Comp. Immunol.** 41(3):447-453.

Kencana, G.A.Y., I.M. Kardena, and I.G.N.K. Mahardika. 2012. Peneguhan diagnosis penyakit Newcastle disease lapang pada ayam buras di Bali menggunakan teknik RT-PCR. **J. Ked. Hewan.** 6(1):28-31.

Kim, L.M., C.L. Afonso, and D.L. Suarez. 2006. Effect of probe-site mismatches on detection of virulent newcastle disease viruses using a fusion-gene real-time reverse transcription polymerase chain reaction test. **J. Vet. Diagn. Invest**. 18(6):519-528.

Kim, L.M., D.J. King, D.L. Suarez, C.W. Wong, and C.L. Afonso. 2007. Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. **J. Clin. Microbiol.** 45(4):1310-1314.

Kim, L.M., D.L. Suarez, and C.L. Afonso. 2008. Detection of a broad range of class I and II Newcastle disease viruses using a multiplex real-time reverse transcription polymerase chain reaction assay. **J. Vet. Diagn. Invest.** 20(4):414-425.

Lomniczi, B., E. Wehmann, J. Herczeg, A. Ballagi-Pordany, E.F. Kaleta, O. Werner, G. Meulemans, P.H. Jørgensen, A.P. Mante, A.L.J. Gielkens, I. Capua, J. Damoser, Lomniczi B, Wehmann E, Herczeg J, Ballagi-Pordany A, Kaleta EF, Werner O, Meulemans G, P.H. Jørgensen, A.P. Mante, A.L.J. Gielkens, I. Capua, and J. Damoser. 1998. Newcastle disease outbreaks in recent years in Western Europe were caused by an old (VI) and a novel genotype (VII). **Arch. Virol.** 143(1):49-64.

Miller, P.J., C.L. Afonso, J. El Attrache, K.M. Dorsey, S.C. Courtney, Z. Guo, and D.R. Kapczynski. 2013. Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. **Dev. Comp. Immunol**. 41(4):505-513.

Oberdorfer, A. and O. Werner. 1998. Newcastle disease virus: Detection and characterization by PCR of recent German isolates differing in pathogenicity. **Avian Pathol.** 27(3):237-243.

OIE. 2012. **OIE Terrestial Manual Newcastle Disease**. Office International des Epizooties.

Rout, S.N. 2007. The role of Newcastle disease virus internal protein in pathogenesis. **Desertation**. Palce Published. University of Maryland. College Park, Maryland, US.

Saepulloh, M. and Darminto. 2005. Kajian Newcastle disease pada itik dan pengendaliannya. **Wartazoa**. 15(2):84-94.

Sa'idu, L. and P. Abdu. 2008. Outbreak of Viscerotropic Velogenic form of Newcastle disease in vaccinated six week old pullet. **Sokoto J. Vet. Sci**. 7(1):37-40.

Tarmudji. 2005. Penyakit pernafasan pada ayam ditinjau dari aspek klinik dan patologik serta kejadiannya di Indonesia. **Wartazoa**. 15(2):72-83.

Wise, M.G., D.L. Suarez, B.S. Seal, J.C. Pedersen, D.A. Senne, D.J. King, D.R. Kapczynski, and E. Spackman. 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. **J. Clin. Microbiol**. 42(1):329-338.