Reverse transcription PCR-based detection of matrix and hemagglutinin-neuraminidase genes among *Avian orthoavulavirus 1* clinical isolates in the Philippines, 1991-2017

Tolentino Jarel Elgin M.[1,2*], Angeles Jorge Gil C.[3], Galay Remil L.[4] and Umali Dennis V.[5]
1. Graduate School, University of the Philippines-Los Baños, 4031, Laguna, PHILIPPINES
2. Institute of Biology, University of the Philippines-Diliman, 1101, Quezon City, PHILIPPINES
3. Philippine Genome Centre-Agriculture, Office of the Vice-Chancellor for Research and Extension, University of the Philippines-Los Baños, 4031, Laguna, PHILIPPINES
4. Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines-Los Baños, 4031, Laguna, PHILIPPINES
5. Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of the Philippines-Los Baños, 4031, Laguna, PHILIPPINES
*jmtolentino4@up.edu.ph

Abstract
Newcastle disease (ND) is a highly infectious disease that affects devastatingly the avian population worldwide. It is caused by Avian orthoavulavirus 1 (AOAV-1), or better known as Newcastle disease virus belonging to phylum Negarnaviricota, class Monjiviricetes, order Mononegavirales and family Paramyxoviridae. This virus consists of six principal structural proteins namely: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and the large protein RNA dependent RNA polymerase (L).

The present study aimed to molecularly detect the M and HN gene segments of the AOAV-1 field isolates from clinical cases in the Philippines from 1991 through 2017. RT-PCR amplification and sequence analyses using primers NDV-For4359 and NDV-Rev4788 which anneal to the matrix gene and primers NDV-For6369 and NDV-Rev6598 targeting the HN genes, identified all isolates to be AOAV-1. Determining the different genes of the virus would greatly help scientists and researchers to accurately identify the viral isolates in order to improve epidemiological studies and surveillance of the disease in the country.

Keywords: Newcastle disease, matrix, hemagglutinin-neuraminidase.

Introduction

Newcastle disease (ND) caused by *Avian orthroavulavirus*-1 (AOAV-1) is a long-known disease affecting detrimentally the avian population worldwide. This disease was first documented in Jakarta, Indonesia[10]and Newcastle-upon-Tyne, England in 1926[5]. The naming of the disease, Newcastle disease, got its first name to refer to the place of the outbreak to avoid disease confusion with other viral infections[6]. This disease has been subjected to numerous discussions as the same outbreak was observed in the deaths of all chickens in Scotland in 1896 with the probable cause being AOAV-1[14]. Since then, the virus has spread across the globe infecting the bird population[2].

In the Philippines, the first clinical cases of ND were reported in Manila in 1927[20]. It was reported that the disease was highly contagious and had killed at least 50,000 fowls with chickens being the most affected. The fowls usually died within 1 to 7 days following the onset of symptoms. The results of the cases have shown that the disease is essentially an infection of the alimentary tract which is one of the clinical symptoms of ND in today's characterization[12].

Based from the molecular characteristics of AOAV-1, this virus is an enveloped, single-stranded, non-segmented, negative sense RNA virus. Its genome is consisting of six open reading frames and encodes for six principal structural proteins namely: the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and the large protein, RNA dependent RNA polymerase (L). In particular, M and HN proteins possess essential functions for AOAV-1 virulence. M protein has a vital role in the AOAV-1 assemblage[4]. Based on its nucleotide sequence analysis, this protein comprises of 365 amino acids with a molecular weight of 40kDa[13,25].

This protein is demonstrated to be a nuclear cytoplasmic shuttling protein and ultimately functions for the assembly and budding of the viral particles in the virus' cytoplasm[8,17]. The M protein is also responsible for the localization in the virus' nucleus which is known to control AOAV-1 replication and transcription[7]. HN protein has an important role in viral proliferation through binding to sialic acid residues on the host membrane producing infection to the host[15].

This protein possesses two functions in the virus namely hemagglutinin and neuraminidase activities[25]. Hemagglutinin is produced via the absorption of the virus by the cell receptors of the host's red blood cells[9] while the neuraminidase activity is caused by the hydrolyzation of the

ketosidic bonds between neuraminic acids on the receptor of the hosts, allowing the F protein to make contact and penetrate the cell membrane of the host[11]. This only shows that M and HN genes are essential in AOAV-1 pathogenicity and replication and by correctly identifying this disease agent using these genes, this will be valuable in creating control and prevention strategies against the disease.

Thus, the main objective of the present work was to detect molecularly the M and HN genes among AOAV-1 field isolates in the Philippines from the year 1991-2017 using reverse-transcriptase polymerase chain reaction. Additionally, it also aimed to compare the AOAV-1 sequences in this study to consensus reference sequences of AOAV-1 obtained in the GenBank.

Material and Methods

AOAV-1 viral strains: Ten AOAV-1 field strains isolated from different infection farm locations situated in northern Philippines (Rizal, Pampanga, Bulacan and Tarlac) and southern Philippines (Zamboanga del Sur, Davao del Sur and Davao City) in different infection time (1991, 2014, 2015, 2016, 2017) were used to detect M and HN genes among ND isolates in the Philippines. The oldest strain PDU19KVG1 was isolated from Rizal in 1991 that infected the duck species. The second strain ZLY14KVG2 was isolated from Zamboanga Del Sur in 2014 that infected the layer flock. The third strain PLY15KVG3 was isolated from Pampanga in 2015 that infected the layer flock. The fourth strain DLY15KVG4 was isolated from Davao del Sur in 2015 that infected the layer flock.

The fifth strain PBR15KVG5 was isolated from Pampanga that infected the broiler flock. The sixth strain BLY16KVG6 was isolated from Bulacan in 2016 that infected the layer flock. The seventh strain TLY16KVG7 was isolated from Tarlac in 2016 that infected the layer flock. The eighth strain TLY17KVG8 was isolated from Tarlac in 2017 that infected the layer flock. The ninth strain DBR17KVG9 was isolated from Davao City in 2017 that infected the broiler flock. The tenth strain RPG17KVG10 was isolated from Rizal that infected the pigeon species in 2017.

The samples obtained were dependent on the willingness of the affected farms to participate in the study. Internal organs collected from these avian species consist of trachea, liver, lungs, spleen, kidneys and cecal tonsils. The samples were stored in individual air-tight plastic containers and stored at -20ºC until analysis.

Reverse-transcriptase PCR (RT-PCR) and PCR re-amplification: Tissue samples were pooled as previously described[24]. RNA extraction was performed using QIAamp® Viral RNA Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol. The extracted RNA was reverse transcribed to complementary DNA using random hexamers and Primescript® reverse transcriptase (Takara Bio-Inc, Shiga, Japan) and the cDNA was stored at -80 °C until analysis. The primers used to target the M gene are NDV-For4359 (5'- GTGACAGGCAAYCTTGATA TATC- 3') and NDV-Rev4788 (5'-CTTGTAGTGGC TCTCATCTG-3'). For the HN gene, the primers used are NDV-For6369 (5'- AGGCYTCACAACATCYGTTC-3') and NDV-Rev6598 (5' TYGATATGCCTRCGAGRTCG-3')[23].

RT-PCR procedure was conducted by using SapphireAmp® Fast PCR Master Mix (Takara Bio) as the precast mix, 5 uM of forward and reverse primers targeting the M and HN genes and 50ng of the cDNA to be used as the template[24]. RT-PCR amplification was performed using the following thermocycling conditions: initial denaturation at 95 ℃ for 2 min followed by denaturation at 35 cycles of 98 ℃ for 10 s, annealing at 55 ℃ for 10 s, extension at 72 ℃ for 10–20 s and a final extension at 72 ℃ for 2 min. After the completion of the RT-PCR amplification, 12.5µl of the reaction mixture was aliquoted onto an agarose gel electrophoresis at 1.2% stained with 0.8µl of ethidium bromide. The gel was subsequently visualized in GenoSens 1869 Gel Documentation and Analysis System (Clinx Science Instruments Co. Ltd, Shanghai, China).

For checking of purity and concentration of the amplified cDNA products, DNA concentration readings using the Nanodrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) were acquired. Viral cDNA products were purified using GeneJet® Gel Extraction Kit (Thermo Fisher Scientific, Massachusetts, USA). The purified viral cDNA products from the first round of PCR amplification were used as the template for the second of PCR re-amplification of the viral cDNA to increase the concentration of the products. Finally, the purified cDNA products were sent to Macrogen Inc. (Seoul, South Korea) for nucleotide sequencing of both the forward and reverse reactions.

Nucleotide sequencing analysis: The nucleotide sequences of M and HN were analyzed using CodonCode Aligner® (version 7.0.1, CodonCode Corporation, MA)[19] and Clustal Omega (version 1.2.4, Conway Institute UCD Dublin, Ireland)[21]. Confirmation of homology and identity was conducted using Nucleotide Basic Local Alignment Search Tool or BLASTn[3]. The analyzed sequences were compared with consensus sequences of AOAV-1 reference strains obtained from GenBank. Pairwise and multiple sequence alignments were performed using Clustal Omega version 1.2.4.

Results and Discussion

Various farms from different time periods in the Philippines reported infections suspected to be ND. The first flock was from a duck farm located in Rizal province situated in northern Philippines. The viral sample (PDU91KVG1) from this flock was obtained in 1991 and the collected samples were stored in the College of Veterinary Medicine in UPLB. Information on the farm size, the size of the affected flock,

mortality rate and clinical signs were not made available. The second flock was from a layer farm located in Zamboanga del Sur situated in southern Philippines. The viral sample (ZLY14KVG2) from this flock was obtained in an outbreak in 2014. The farm size has a total capacity of 180,000 and the reported size of the affected flock is 31,625. The reported mortality rate is 61%.

The clinical signs observed are the following: depression, decrease in feed intake, conjunctivitis, facial swelling, greenish diarrhea, opisthotonos and torticollis. The third flock was from a layer farm in Pampanga province situated in northern Philippines. The sample (PLY15KVG3) from this flock was obtained in an outbreak in 2015. The farm size has a total capacity of 40,000 and the reported size of the affected flock is 12,000.

The reported mortality rate is 15%. The clinical signs observed are the following: gasping, conjunctivitis, periorbital swelling, lethargy, greenish diarrhea, torticollis, hemorrhagic proventriculus and cecal tonsils. The fourth flock was from a layer farm located in Davao del Sur situated in southern Philippines. The viral sample (DLY15KVG4) from this flock was obtained in an outbreak in 2015. The clinical signs observed are the following: facial swelling, conjunctivitis, nasal discharge and proventriculitis. Information on the farm size, the size of the affected flock and the reported mortality rate were not made available.

The fifth flock was from a broiler farm located in Pampanga situated in northern Philippines. The viral sample (PBR15KVG5) from this flock was obtained in an outbreak in 2015. Information on the farm size, the size of the affected flock, mortality rate and clinical signs were not made available. The sixth flock was from a layer farm in Bulacan situated in northern Philippines. The viral sample (BLY16KVG6) from this flock was obtained in an outbreak in 2016. The farm size has a total capacity of 100,000 and the reported size of the affected flock is 30,000.

The reported mortality rate is 41%. The clinical signs observed are the following: conjunctivitis, swollen head, tracheal rales, nasal discharges and torticollis. The sixth flock was from a layer farm in Tarlac situated in northern Philippines. The viral sample (TLY16KVG7) from this flock was obtained in an outbreak in 2016. The clinical signs observed are the following: periorbital swelling, lacrimation, transparent mucoid nasal and mouth discharge, brilliant green diarrhea, depression, paresis, ruffled feathers, gasping and incoordination. Information on the farm size, the size of the affected flock and the reported mortality rate were not made available.

The eight flock was from a layer farm in Tarlac situated in northern Philippines. The viral sample (TLY17KVG8) from this flock was obtained in an outbreak in 2017. The farm size has a total capacity of 120,000 and the reported size of the affected flock is 40,000. The clinical signs observed are high mortalities in 11-week old pullets. The ninth flock was from a broiler farm in Davao City situated in southern Philippines. The viral sample (DBR17KVG9) from this flock was obtained in an outbreak in 2017. The farm size has a total capacity of 120,000 and the reported size of the affected flock is 40,000.

The reported mortality rate is 30%. The clinical signs observed are the following: sudden increase in mortalities, greenish whitish diarrhea and torticollis. The tenth flock was from a pigeon population in Rizal province situated in northern Philippines. The farm size has a total capacity of 600 and the reported size of the affected flock is 600. The reported mortality rate is 10%. The clinical signs observed are the following: respiratory signs, ocular and nasal discharges and torticollis. The differences in reported mortality and clinical signs observed in the flocks in various farm locations may be described by different internal and external compounding factors such as host range, susceptibility of the host, immune status and environmental distress due to differing climatic phenomena like age of the affected species, vaccination dosage and history and the spread of exposure which may greatly influence the severity of ND[24].

Determination of M and HN genes is important as it will help scientists and researchers to accurately identify AOAV-1 to improve epidemiological studies and surveillance of the disease in the country.

Table 1
Summary of the detected M and HN genes in the Philippine field AOAV-1 clinical isolates.

Official viral isolate names	M gene	HN gene
1. DBR17KVG9	+	-
2. PLY15KVG3	+	+
3. PBR15KVG5	-	-
4. DLY15KVG4	+	-
5. BLY16KVG6	+	+
6. PDU91KVG1	-	+
7. ZLY14KVG2	+	-
8. TLY17KVG8	+	-
9. TLY16KVG7	+	+
10. RPG17KVG10	+	-

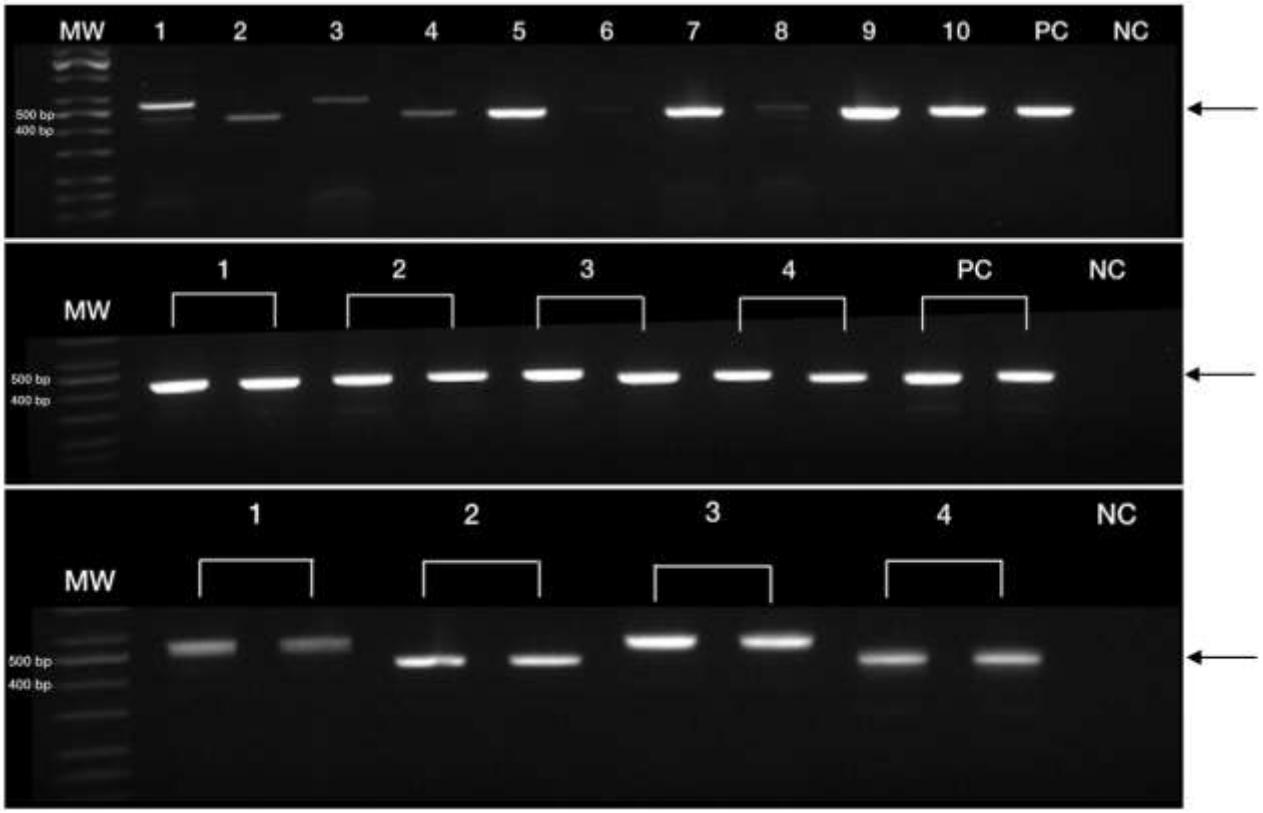

Fig. 1: Agarose gel electrophoresis of RT-PCR products of M gene. (A) PCR products amplified from the partial M gene using primer A (NDV-For4359 and NDV-Rev4788). Lane MW: KAPPA Ladder molecular weight marker; Lane 1: DBR17KVG9; Lane 2: PLY15KVG3; Lane 3: PBR15KVG5; Lane 4: DLY15KVG4; Lane 5: BLY16KVG6; Lane 6: PDU91KVG1; Lane 7: ZLY14KVG2; Lane 8: TLY17KVG8; Lane 9: TLY16KVG7; Lane 10: RPG17KVG10; Lane 11: Positive control (La Sota); Lane 12: Negative control; (B) PCR re-amplification of the cDNA products of (A) with higher concentration; Lane MW: KAPPA Ladder molecular weight marker; Lane 1 and 2: BLY16KVG6; Lane 3 and 4: ZLY14KVG2; Lane 5 and 6: TLY16KVG7; Lane 7 and 8: RPG17KVG10; Lane 9 and 10: Positive control; Lane 11: Negative control; (C) PCR re-amplification of the cDNA products of (A) with higher concentration; Lane MW: KAPPA Ladder molecular weight marker; Lane 1 and 2: DBR17KVG9; Lane 3 and 4: PLY15KVG3; Lane 5 and 6: TLY17KVG8; Lane 7 and 8: DLY15KVG4; Lane 10: Negative control

Table 1 presents the summary of the detected M and HN genes in different AOAV-1 isolates in the Philippines. Figure 1 shows the agarose gel electrophoresis result of the partial M gene as parallel to the positive control. The expected band size of the amplicons is 429bp.

Figure 1a shows that DBR17KVG9, PLY15KVG3, DLY15KVG4, BLY16KVG6, ZLY14KVG2, TLY17KVG8, TLY16KVG7 and RPG17KVG10 are all positive for AOAV-1 coding for the matrix gene while PBR15KVG5 shows multiple banding patterns and PDU91KVG1 shows no amplicons. Figures 1b and 1c demonstrate the PCR re-amplification of the detected bands in figure 1a.

Al-Habeeb et al[1] successfully detected and characterized matrix genes from AOAV-1 strains using real time RT-PCR and melting curve analysis. Rahman et al[18] molecularly detected matrix genes of AOAV-1 strains circulating in Bangladesh using real time RT-PCR amplification. Figure 2 shows the agarose gel electrophoresis result of the partial HN gene as parallel to the positive control. The expected band size of the amplicons is 229bp. Figure2a shows that PLY15KVG3, BLY16KVG6, TLY16KVG7 and PDU91KVG1 are all positive for AOAV-1 coding for the HN gene while DBR17KVG9, PBR15KVG5, DLY15KVG4, ZLY14KVG2, TLY17KVG8 and RPG17KVG10 show no amplicons. Figure 2b and 2c demonstrate the PCR re-amplification of the detected bands in figure 2a.

Otim et al[16] they reported the molecular characterization and construction of phylogenetic tree of AAV1 field isolates in Eastern Uganda by comparing the nucleotide sequences using the HN protein genes.

Triosanti et al[22] detected and molecularly characterized the HN gene fragments of Indonesian AAOV1 isolates from vaccinated farms and constructed phylogenetic tree confirming the isolates to be ND. Table 2 shows the similarity of the M and HN gene sequences of this study to the AOAV-1 reference strains in the GenBank. This

confirmation provides evidence of the identity and similarity of the isolated strains to be AOAV-1. Our results suggest that detecting and characterizing M and HN genes can aid in correctly identifying this disease agent which will give us knowledge on creating more specific control strategies against the disease.

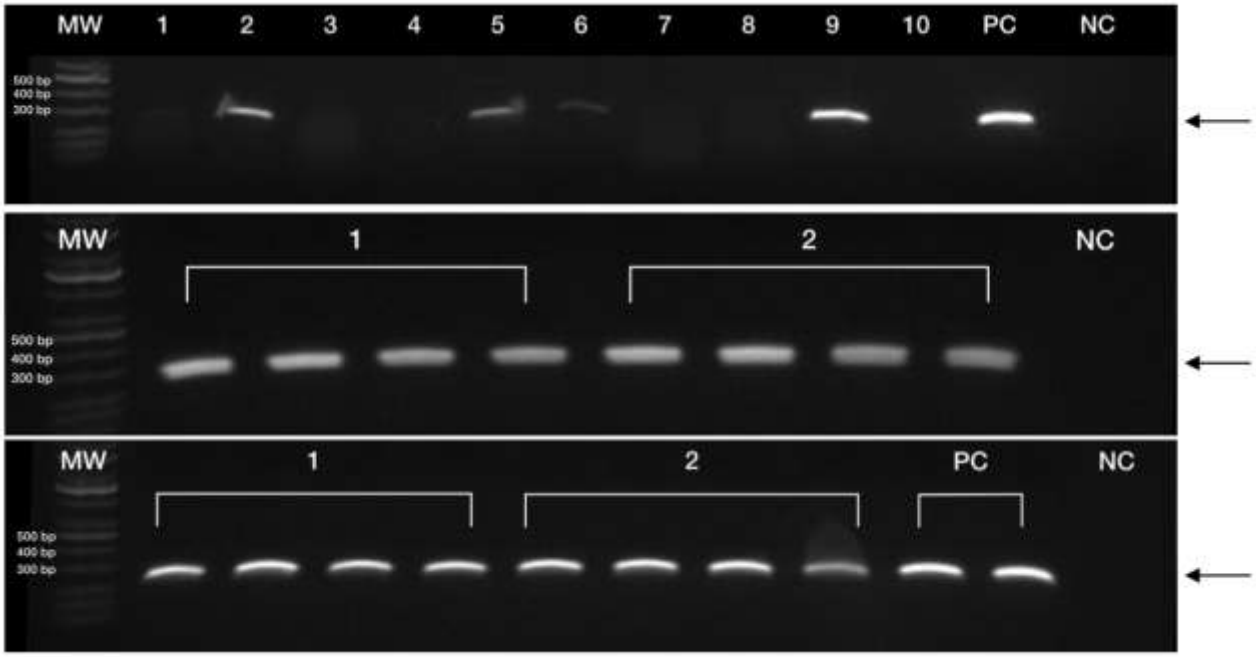

Fig. 2: Agarose gel electrophoresis of RT-PCR products of HN gene. (A) PCR products amplified from the partial HN gene using primer E (NDV-For6369 and NDV- Rev6598). Lane MW: KAPPA Ladder molecular weight marker; Lane 1: DBR17KVG9; Lane 2: PLY15KVG3; Lane 3: PBR15KVG5; Lane 4: DLY15KVG4; Lane 5: BLY16KVG6; Lane 6: PDU91KVG1; Lane 7: ZLY14KVG2; Lane 8: TLY17KVG8; Lane 9: TLY16KVG7; Lane 10: RPG17KVG10; Lane 11: Positive control (La Sota); Lane 12: Negative control; (B) PCR re-amplification of the cDNA products of (A) with higher concentration; Lane MW: KAPPA Ladder molecular weight marker; Lane 1, 2, 3, 4: BLY16KVG6; Lane 5, 6, 7, 8: PDU91KVG1, Lane 9: Negative control; (C) PCR re- amplification of the cDNA products of (A) with higher concentration; Lane MW: KAPPA Ladder molecular weight marker; Lane 1, 2, 3, 4: PLY15KVG3; Lane 5, 6, 7, 8: TLY16KVG7, Lane 9 and 10: Positive control, Lane 11: Negative control

Table 2
Nucleotide sequence similarity of the Philippine field AOAV-1 clinical isolates using the M and HN gene sequences.

	M gene								**HN gene**					
Residue Number (4403-6497)	**4407**	**4416**	**4434**	**4441**	**4455**	**4467**	**4475**	**4497-4502**	**6430**	**6431**	**6451**	**6470**	**6477**	**6490-6497**
Consensus[a]	G	C	G	C	T	A	T	AAAAAA	G	T	A	A	A	TTCCGGAT
DBR17KVG9	+[b]	+	A	+	+	+	+	+	-[d]	-	-	-	-	-
PLY15KVG3	+	T	+	T	+	+	+	+	+	+	+	+	+	+
DLY15KVG4	+	T	+	T	+	+	+	+	-	-	-	-	-	-
BLY16KVG6	+	+	A	+	+	+	+	+	+	+	+	+	T	±[c]
PDU91KVG1	+	+	A	+	+	+	+	+	+	+	+	+	+	+
ZLY14KVG2	+	+	A	+	+	C	+	+	-	-	-	-	-	-
TLY17KVG8	+	+	A	+	+	+	+	+	-	-	-	-	-	-
TLY16KVG7	+	+	A	+	+	+	+	+	+	+	+	+	+	+
RPG17KVG10	+	T	+	T	+	+	+	+	-	-	-	-	-	-
Positive control	+	+	A	+	+	+	+	+	-	-	-	-	-	-

[a] The consensus nucleotide sequence was derived from the whole genome sequence of M and HN genes of AOAV-1 strains from GenBank
[b] Same as the consensus sequence
[c] Different from the consensus sequence
[d] Not detected

Conclusion

ND is a long-known disease in the country and still poses a big threat to the avian population and to the economy. Therefore, more effective control and preventive strategies such as the use of vaccines must be performed to mitigate the effects of this debilitating disease. In order to form these strategies, detection of many genes of the disease will be helpful with this approach.

In this study, M and HN genes were successfully detected in AOAV-1 field strains from 1991-2017 using the RT-PCR method. This implies that the method used is a powerful tool in detecting the viral samples even if the samples were stored for a very long time. This test will be useful for the screening of the suspected AOAV-1 samples which will be valuable for the surveillance and epidemiological investigation of the disease in the country.

Acknowledgement

The authors would like to thank Dr. Ian Kendrich Fontanilla and all the laboratory members of DNA Barcoding Laboratory, Institute of Biology, UP Diliman for their kind assistance and for allowing the primary author to use the laboratory reagents and equipment. The authors would also like to thank the Department of Science and Technology - Accelerated Science and Technology Human Resource Development Program (DOST-ASTHRDP) grant for funding of this research.

References

1. Al-Habeeb M.A., Mohamed M.H.A. and Sharawi S., Detection and characterization of Newcastle disease virus in clinical samples using real time RT-PCR and melting curve analysis based on matrix and fusion genes amplification, *Vet. World*, **6(5)**, 239-243 **(2013)**

2. Brown V.R. and Bevins S.N., A review of virulent Newcastle disease viruses in the United States and the role of wild birds in viral persistence and spread, *Vet. Res.*, **48(1)**, 68 **(2017)**

3. Camacho C., Coulouris G., Avagyan V., Ma N., Papadopoulos J., Bealer K. and Madden T.L., BLAST+: architecture and applications, *BMC Bioinformatics*, **10**, 421 **(2009)**

4. Chambers P., Millar N.S. and Emmerson P.T., Nucleotide sequence of the gene encoding the fusion glycoprotein of Newcastle disease virus, *J. Gen. Virol.*, **67(12)**, 2685-2694 **(1986)**

5. Doyle T.M., A hitherto unrecognized disease of fowls due to a filter-passing virus, *J. Comp. Pathol*., **40**, 144-169 **(1927)**

6. Doyle T.M., Newcastle disease of fowls, *J. Comp. Pathol*., **48**, 1-20 **(1935)**

7. Duan Z., Chen J., Xu H., Zhu J., Li Q., He L. and Liu X., The nucleolar phosphoprotein B23 targets Newcastle disease virus matrix protein to the nucleoli and facilitates viral replication, *Virology*, **452**, 212-222 **(2014)**

8. Harrison J.L. and Alders R.G., An assessment of chicken husbandry including Newcastle disease control in rural areas of Chibuto, Mozambique, *Trop. Anim. Health Prod.*, **42(4)**, 729-736 **(2010)**

9. Kimball J.W., Introduction to Immunology, 3rd edition, New York, Macmillan Publishing Company, 523 **(1990)**

10. Kraneveld F.C., A poultry disease in the Dutch East Indies, *Ned. Indisch. Bl. Diergeneeskd.*, **38**, 448-450 **(1926)**

11. Lamb R.A. and Kolakofsky D., Paramyxoviridae: the viruses and their replication, In Fields B.N., Knipe D.M. and Howley P.M., Eds., Fields virology 3rd edition, Vol. 1, Philadelphia, PA, Lippincott-Raven, 1177-1203 **(1996)**

12. Lee D.H., Kwon J.H., Noh J.Y., Park J.K., Yuk S.S., Erdene-Ochir T.O. and Song C.S., Viscerotropic velogenic Newcastle disease virus replication in feathers of infected chickens, *J. Vet. Sci*., **17(1)**, 115-117 **(2016)**

13. Liu Q., Mena I., Ma J., Bawa B., Krammer F., Lyoo Y.S. and García-Sastre A., Newcastle disease virus-vectored H7 and H5 live vaccines protect chickens from challenge with H7N9 or H5N1 avian influenza viruses, *J. Virol.*, **89(14)**, 7401-7408 **(2015)**

14. Macpherson L.W., Some observations on the epizootiology of Newcastle disease, *Can. J. Comp. Med. Vet. Sci.*, **20(5)**, 155 **(1956)**

15. Millar N.S., Chambers P. and Emmerson P.T., Nucleotide sequence analysis of the haemagglutinin-neuraminidase gene of Newcastle disease virus, *J. Gen. Virol.*, **67(9)**, 1917-1927 **(1986)**

16. Otim M.O., Christensen H., Jørgensen P.H., Handberg K.J. and Bisgaard M., Molecular characterization and phylogenetic study of Newcastle disease virus isolates from recent outbreaks in eastern Uganda, *J. Clin. Microbiol*., **42(6)**, 2802-2805 **(2004)**

17. Pantua H.D., Mcginnes L.W., Peeples M.E. and Morrison T.G., Requirements for the assembly and release of Newcastle disease virus-like particles, *J. Virol*., **80(22)**, 11062-11073 **(2006)**

18. Rahman M., Barman L., Chowdhury E. and Islam M., Detection of Newcastle disease virus of poultry by real time reverse transcription-polymerase chain reaction, *Bangladesh Veterinarian*, **33(1)**, 16-22 **(2017)**

19. Richterich P., New Products: CodonCode Aligner Version 1.2 Released, *Genet. Med*., **6**, 162-163 **(2004)**

20. Rodier E.A., Philippine fowl disease, *Proc. Soc. Exp. Biol. Med*., **25(9)**, 781–783 **(1928)**

21. Sievers F. and Higgins D.G., Clustal Omega, *Curr. Protoc. Bioinform*., **48**, 1.25.1-1.25.33 **(2014)**

22. Triosanti L.S., Wibowo M.H. and Widayanti R., Molecular characterization of hemagglutinin-neuraminidase fragment gene of Newcastle disease virus isolated from periodically-vaccinated farms, *Vet. World*, **11(5)**, 657 **(2018)**

23. Umali D.V., Katoh H., Ito H., Shirota K., Suzuki T. and Ito T., Molecular epidemiology of Newcastle disease virus isolates from vaccinated commercial poultry farms in non-epidemic areas of Japan, *Virol. J.*, **10(1)**, 330 **(2013)**

24. Umali D.V., Lopez A.L.M., Torres M.I.P., Umandal M.C. and Katoh H., Molecular Characterization and Phylogenetic Analysis of Newcastle Disease Virus Isolated from Clinical Cases of Newcastle Disease in Commercial Layer Farms in the Philippines, *Philipp. J. Vet. Med.*, **54(1)**, 17-26 **(2017)**

25. Yusoff K. and Tan W.S., Newcastle disease virus: macromolecules and opportunities, *Avian Pathol*., **30(5)**, 439-455 **(2001)**.

(Received 12th June 2020, accepted 14th August 2020)