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# Molecular characterization of a Newcastle disease virus isolate from a diseased chicken in the Philippines in 2017

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**Abstract**

**Newcastle disease (ND) is one of the most serious diseases affecting numerous avian species worldwide. However, data on the genetic characterization of ND virus (NDV) in the Philippines are limited. Herein, the complete sequences of the fusion (*F*) and hemagglutinin-neuraminidase (*HN*) genes of a NDV strain isolated in Central Luzon in 2017 were genetically analyzed. The results revealed that the field isolate belonged to subgenotype VII.2 and the genome encoded the amino acid sequence of the F0 protein proteolytic cleavage site motif ($^{112}$R-R-Q-K-R-F$^{117}$), indicating that it was a virulent strain. The current NDV strain was genetically related to Indonesian and Israeli NDV strains. Some amino acid substitutions were found in the transmembrane domain and neutralizing epitopes of the HN protein.**

Key Words: Genotype VII, Newcastle disease virus, The Philippines

The most notable avulavirus in the genus *avian orthoavulavirus 1*, formerly designated as *avian avulavirus 1*, is *avian paramyxoviruses 1* or Newcastle disease virus (NDV), which causes disease in a wide range of both domestic and wild birds worldwide[4]. The NDV genome contains six open reading frames that encode the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), large RNA-dependent polymerase protein (L), and an additional protein V that is expressed by RNA editing of P mRNA[1,24].

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Newcastle disease (ND), which is caused by a virulent form of NDV, is one of the most economically important diseases in the poultry industry. Virulent NDV strains are defined by the criteria of the World Organization for Animal Health as having an intracerebral pathogenicity index of ≥0.7 or a fusion cleavage site sequence with multiple basic amino acids (aa) ($^{112}$R/K/G-R-Q/K-K/R-R-F$^{117}$)[17]. Within a single serotype, NDV strains can be divided into class I, which consists of only genotype I, and class II, which consists of at least 21 genotypes, I to XXI[4]. Class II genotype VII viruses, which likely emerged in the Far East in the 1990s and subsequently spread to Asia, Europe, and Africa, have been recently reported as the prevalent virulent strains of NDV in Asia[6,30]. At present, genotype VII viruses are classified into three subgenotypes: VII.1.1, VII.1.2, and VII.2. More specifically, subtype VII.1.1 is a combination of the former subgenotypes VIIb, VIId, VIIe, VIIj, and VIIl. The separate subgenotype VII.1.2 consists of only the former subgenotype VIIf. The subgenotype VII.2 includes former VIIa, VIIh, and VIIi[4].

There have been only two reports of ND outbreaks among commercial chicken farms in the Philippines. These outbreaks caused significant economic losses to the affected farms by way of increased mortality. Phylogenetic analyses of partial *F* gene sequences (766 bp) indicated that subgenotypes VIIa, VIIh, and VIIi had circulated in chicken flocks from 2014 to 2016 in the Philippines[2,28]. The aim of the present study was to molecularly characterize the full-length *F* and *HN* genes of a NDV strain isolated from a diseased layer chicken in commercial farm in Nueva Ecija, Central Luzon, Philippines.

Three chicken lung samples were collected by the Regional Animal Disease Diagnostic Laboratory (Department of Agriculture Field Office III, San Fernando, Pampanga, Philippines) under institutional approval and permission from the chicken owners.

One lung sample was collected from a 30-week-old layer chicken involved in a suspected outbreak of NDV in Nueva Ecija in 2017 and then transferred to Obihiro University of Agriculture and Veterinary Medicine in accordance with the agreement between the two parties. The sample was homogenized in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with kanamycin (1 mg/ml), gentamycin (100 µg/ml), and amphotericin B (5 µg/ml) to prepare 30% homogenates. A volume of 200 µl of tissue supernatant was then inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs following the recommendations of the World Organization for Animal Health[17]. All eggs were incubated at 37°C for up to 5 days with daily candling and inspections for embryonic death. Allantoic fluid was harvested from the inoculated eggs and subjected to the hemagglutination (HA) assay using 0.5% chicken red blood cells. All HA-positive samples were confirmed for the presence of NDV using a hemagglutination inhibition (HI) test and conventional reverse transcription polymerase chain reaction (RT-PCR).

Total RNA was extracted from the HA-positive allantoic fluid using ISOGEN II reagent (Nippon Gene Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol and reverse transcribed into complementary DNA with the use of random primers (Invitrogen Corporation, Carlsbad, CA, USA) and Moloney murine leukemia virus reverse transcriptase (Invitrogen Corporation) under the following conditions: 25°C for 10 min, 37°C for 50 min, and 65°C for 10 min.

Amplification was conducted by conventional RT-PCR with the use of several sets of primers specific for the NDV genome (Supplemental Table 1) and in accordance with the following thermal conditions: an initial denaturation step at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 52–58°C for 30 s (depending on the primers), and 72°C for 40 s, and a final extension step at 72°C for 10 min.

The PCR products were separated on a 0.5% agarose gel and purified using the GeneClean® II Kit (MP Biomedicals, Santa Ana, CA, USA). The

**Table 1.** Amino acid substitutions in the F protein of a NDV PH1-18 isolate and viruses representing other genotypes within class II

| Virus | F2 subunit (32–116) | F1 subunit (117–533) | | | | |
|---|---|---|---|---|---|---|
| | | 332 | 346 | 390 | Heptad Repeat c (467–502) | Trans-membrane (503–522) |
| Consensus[a] | 82E | E | Y | V | 485N | 522A |
| PH1-18 | D | K | F | I | S | V |
| VII i | -[b] | - | - | - | - | |
| Class II (I-XXI) | -/D[c] | -/Q[d] | - | -/I[e] | - | - |

[a] The consensus amino acid sequence was derived from 180 velogenic, mesogenic, and lentogenic NDV strains from GenBank.
[b] Same as the consensus
[c] Amino acid substitution was found in genotype II and subgenotype VIIg.
[d] Amino acid substitution was found in genotype XXI.
[e] Amino acid substitution was found in genotype VI.

**Table 2.** Amino acid substitutions in the HN protein of a NDV PH1-18 isolate

| Virus | Transmembrane domain (25–45) | N-linked glycosylation sites | Neutralizing epitopes | Other positions 79, 82, 83, 91, 129, 333, 338, 389 |
|---|---|---|---|---|
| Consensus[a] | 33I, 34V, 36T | 119, 341, 433, 481, 508, 538 | 263N, 514I, 569D | D, D, R, E, D, G, K, T |
| PH1-18 | 33M, 34M, 36I | Lost at 508 | 263K, 514V, 569N | V, N, W, V, E, E, R, A |

[a] The consensus amino acid sequence was derived from a total of 100 velogenic, mesogenic, and lentogenic NDV strains retrieved from the GenBank database, including the B1 and La Sota strains as vaccine strains.

full-length *F* and *HN* genes were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

The nucleotide and deduced aa sequences were aligned and analyzed using the Clustal W algorithm of the BioEdit (version 7.2) biological sequence alignment editor[8,26]. The sequence data were analyzed and compared with those of reference NDV strains retrieved from the GenBank database using the Basic Local Alignment Search Tool for nucleotides (BLASTn) (http:www.ncbi.nlm.nih.gov) and the GENETYX (ver. 10) multiple alignment program (Genetyx Corporation, Tokyo, Japan). The reference strains included representatives of all the different NDV genotypes and subgenotypes. Phylogenetic trees of the full-length *F* and *HN* gene sequences were constructed with a maximum likelihood method with 1,000 bootstrap replicates using the General Time Reversible model included with the MEGA6 software package[25]. The nucleotide sequence obtained in this study was deposited into the GenBank database under the accession number MT079856.

Allantoic fluid was harvested from embryonated eggs inoculated with NDV. HA activity was confirmed by the HI test and a partial NDV *F* gene sequence was amplified by conventional RT-PCR, which yielded an amplification product of 403 bp. The isolated NDV strain was designated as Chicken/Philippines/Nueva Ecija/L1513/PH1-18 (abbreviated as strain PH1-18). Interestingly, the NDV strain was isolated from a layer chicken vaccinated against ND, but developed symptoms of nasal discharge, rales, ruffled feathers, pale comb, diarrhea, and

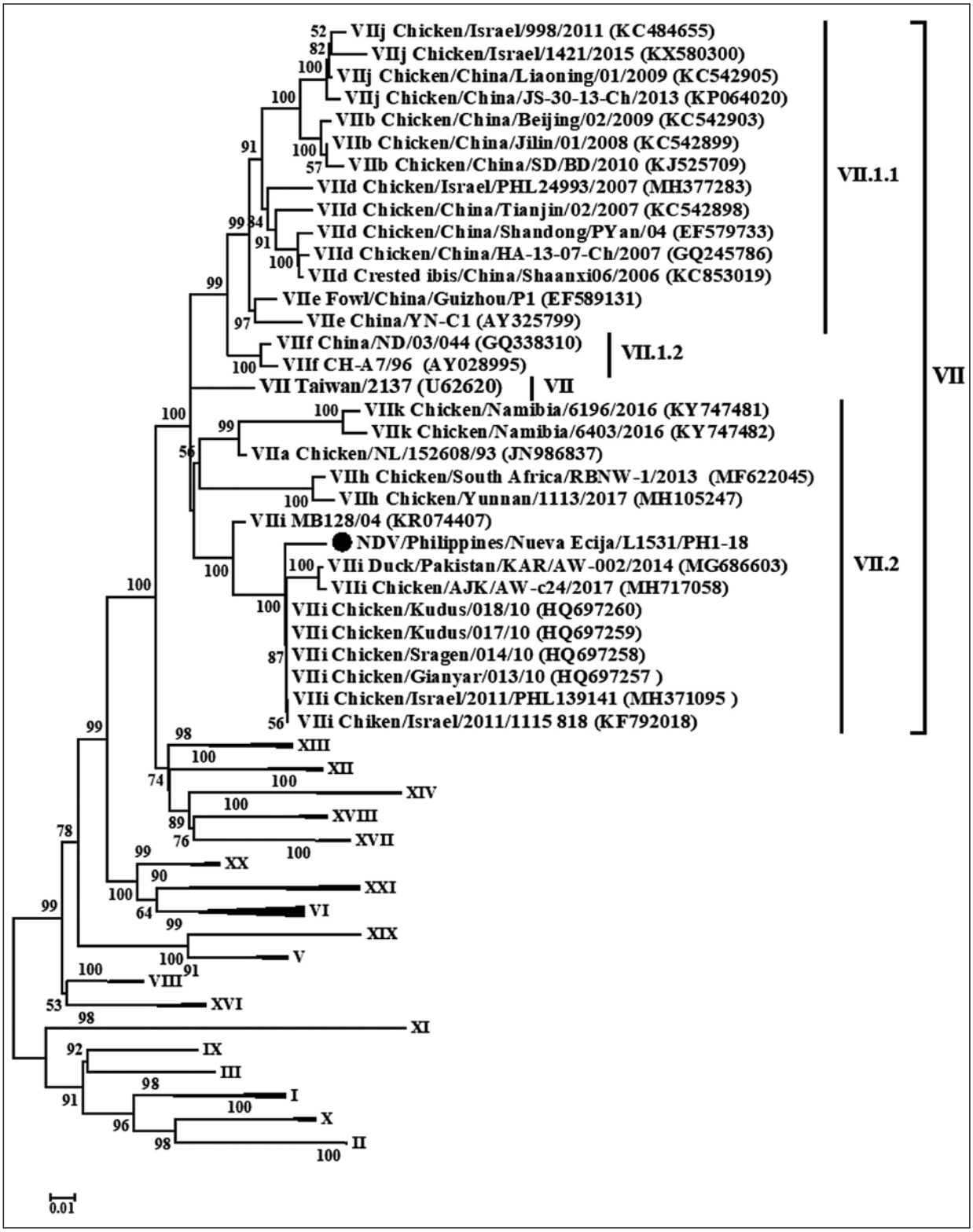


**Fig. 1.** Phylogenetic tree of the full-length sequence of the F gene of a PH1-18 isolate compared with the sequences of other NDV strains downloaded from the GenBank database. The tree was constructed using the maximum likelihood method (1,000 bootstrap replicates) with MEGA6 software. Bootstrap values are shown at the nodes. The sequence determined in this study is marked with a black circle.

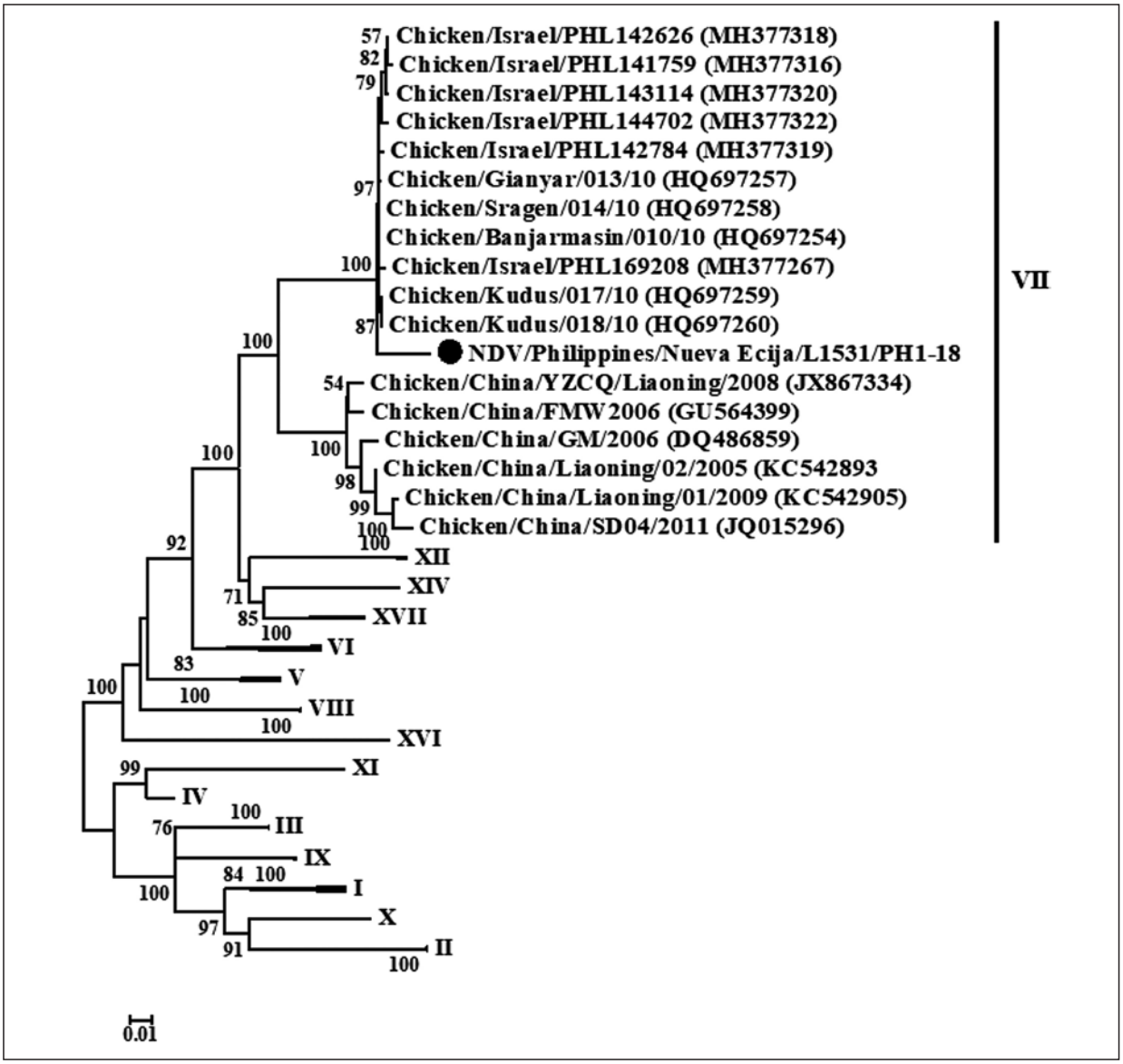


**Fig. 2.** Phylogenetic tree of the full-length sequence of the HN gene of a PH1-18 isolate compared with the sequences of other NDV strains downloaded from the GenBank database. The tree was constructed using the maximum likelihood method (1,000 bootstrap replicates) with MEGA6 software. Bootstrap values are shown at the nodes. The sequence determined in this study is marked with a black circle.

decreased egg production.

The nucleotide and predicted aa sequences of the full-length *F* and *HN* genes and proteins of strain PH1-18 were aligned and analyzed with representative sequences downloaded from the GenBank database for genetic characterization. The results indicated that the full-length *F* and *HN* genes of the current NDV strain shared the highest identity (98.36% nucleotide and 99.64% aa identity) with that of Chicken/Kudus/018/10 (HQ697260) isolated in Indonesia in 2010 and Chicken/Israel/2011/1115 (KF792018) isolated in Israel in 2011.

Analyses of the deduced aa sequence of the F protein of strain PH1-18 revealed a precursor F0 cleavage site motif of $^{112}$R-R-Q-K-R-F$^{117}$. Similar to previous reports[27,31], six potential N-glycosylation sites, 12 cysteine residues, and seven neutralizing

epitopes were conserved in the current NDV strain. However, some aa substitutions were observed in the F1 and F2 subunits of the F protein of strain PH1-18 (Table 1). In particular, strain PH1-18 contained aa substitutions at positions 346 (Y to F), 485 (N to S), and 522 (A to V), which have not been previously reported.

Furthermore, the HN protein of strain PH1-18 consisted of 571 aa residues. The results indicated that the HN protein sequence of strain PH1-18 contained five of six N-linked glycosylation sites, as one site at position 508 was lacking. Some aa substitutions were also observed in the transmembrane domain (I33M, V34M, and T36I), neutralizing epitopes (N263K, I514V, and D569N), and other positions of the HN protein of strain PH1-18 (Table 2).

Phylogenetic analyses of the full-length *F* and *HN* genes of strain PH1-18 and other representative NDV strains were performed to determine the relationship between these viruses. The results indicated that strain PH1-18 belonged to genotype VII (Figs. 1 and 2). Furthermore, a phylogenetic tree of the full-length *F* gene sequence demonstrated that the NDV strain belonged to subgenotype VII.2 (former subgenotype VIIi) (Fig. 1). Also, the results indicated that the *F* gene sequence of strain PH1-18 was closely related to that of the Indonesian (2010) and Israeli (2011) NDV strains.

NDV is an economically important pathogen because it causes high mortality in chicken production. In the Philippines, the first study on molecular characterization of NDV was conducted from 2014 to 2016. The NDV strains from clinically diseased commercial layer and broiler chickens were characterized and tentatively classified to subgenotype VII.2 (former VIIa, VIIh, and VIIi) based on partial sequences of the *F* gene (766 bp). The previous Filipino subgenotype VIIi NDVs were reported to cluster with the Indonesian and Israeli NDV strains isolated in 2010 and 2011, respectively[2,28].

The results of the current study confirmed the continuous circulation NDV of subgenotype VII.2 (former VIIi) among layer chickens in Central Luzon in 2017 based on the phylogenetic and genetic analyses of the full-length *F* and *HN* gene sequences. In particular, strain PH1-18 was classified as a virulent NDV strain based on the aa sequence of the F0 protein proteolytic cleavage site ($^{112}$R-R-Q-K-R-F$^{117}$). Also, the presence of a phenylalanine (F) residue at position 117 and the length of HN protein (571 aa) were determined as characteristic features of the virulent strain[3,17,21].

The results of the current study revealed that strain PH1-18 was closely related to the NDV isolates in Indonesia (2010) and Israel (2011), which is supported by some reports from Pakistan, Israel, and Iran stating that the Indonesian subgenotype VIIi NDVs have a common ancestor[7,14]. The strain PH1-18 appeared to be clustered with the previous Filipino subgenotype VIIi NDV[2,28]. Considering the frequent isolation of NDV subgenotype VIIi in some areas in Central Luzon, live bird markets may possibly be the source of the transmission of NDVs throughout the Philippines[20]. Moreover, live bird markets are considered an important transmission route of NDV and other pathogens[15,16]. Geographically, the Philippines is located in Southeast Asia and is within the migration route of wild birds, known as the East Asia–Australian flyway, which may explain the presence of NDV subgenotype VIIi in the Philippines. In fact, viral transmission between wild birds and poultry has been reported in previous studies[10,23].

Notably, ND outbreaks by virulent genotype VII strains in vaccinated flocks have been recorded in several countries[11,18,19,27,29]. Strain PH1-18 was also isolated from a diseased chicken of a flock vaccinated against ND, although the detailed vaccination protocol was not available for inclusion in this report. The continuously increasing number of ND outbreaks despite vaccination has become a big concern to the ND control programs in countries where ND outbreaks have been recorded. Currently, ND vaccines against avirulent strains (F, B1, La Sota, V4, V4-HR, and I-2) and mesogenic strains

(Mukterswar and Komarov) have been applied against NDV infection[5,17]. In the Philippines, live B1 and La Sota vaccine strains (genotype II) have reportedly been used in commercial layer farms[28]. Strain PH1-18 contained some aa substitutions in the transmembrane domain and the neutralizing epitopes of the HN protein when compared with the vaccine strains (B1 and La Sota) and others. A mutation to the transmembrane domain of the HN protein was considered to affect virus attachment and neuraminidase activity[12]. Regarding the aa substitutions in the neutralizing epitopes, additional data from animal experiments are necessary to identify these mutations, which could lead to neutralization escape variants. Some reports have described the development of genotype VII-matched vaccines as a newer vaccination strategy against ND, which may have a potential for better protection in reducing viral replication and virus shedding than the vaccine using NDV strains belong to genotype II[9,13,22]. However, the exact reason for the occurrence of ND outbreaks in vaccinated flocks should be further investigated. Possible reasons may involve inadequate application of vaccine and biosecurity procedures at the farm level, and genotype mismatching between the vaccine and circulating viruses.

In this study, the aa substitutions at positions 346 (Y to F), 485 (N to S), and 522 (A to V) seemed to be unique to the F protein of strain PH1-18 and might be related to the genetic evolution of NDV or a typical feature distinguishable from other subgenotype VII viruses.

In summary, the circulation of subgenotype VII.2 NDV in the Philippines was compared to previous reports. The genetic characterization of strain PH1-18 provides basic information for further analysis and phenotyping of NDV strains in the Philippines. Further molecular epidemiological analyses of NDVs with larger sample groups are needed to better understand the circulatory patterns of virulent strains of NDV.

## Conflict of interest

The authors have no conflict of interest to declare.

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## Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.69.1.73

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