**Phylogenetic analysis of virulent Newcastle disease virus recently isolated from broiler farms**

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**Abstract**

Newcastle disease (ND) is a highly contagious viral disease that causing high economic losses among poultry farms. The disease is caused by Newcastle disease virus (NDV) that frequently reported in Egypt although of intensive vaccination. Continuous genetic characterization of the virus along with detection of genetic variations in relation to the vaccine strains, are important for proper disease control. Clinical samples were collected from broiler farms in six Egyptian governorates during 2020−2022. NDV was isolated on embryonated chicken egg and identified by real time reverse transcriptase-PCR. Nineteen NDV isolates namely (HS1NDV−HS19NDV) were identified by RT-PCR and deposited on GeneBank database under the accession nos. (OP588159−OP588177). All the isolates found to be velogenic according to the amino acid sequences of the cleavage sits (GRRQKRF). In addition these strains were assigned under genotype VII and sub-genotype VII.1.1. In relation to vaccine strains, comparison of amino acids deduced from partially sequenced fusion gene revealed amino acid substitutions in fusion peptides that are essential in initiation of viral fusion with the host cell membrane. Moreover, HS3NDV had one amino acid substitution that was found in HR1 which shares in formation of the conserved six-helix bundles, that their assembly is tightly joined to the membrane fusion. This study indicates the frequent genetic mutation of NDV that was distinct from vaccine strains.

**Keywords**: Newcastle disease virus, phylogenetic analysis, fusion gene, broiler farms

1. **Introduction**

Newcastle disease (ND) is considered to be one of the highly contagious and economically significant viral diseases that affecting many avian species (Rauw et al., 2009; da Silva et al., 2020; AbdElfatah et al., 2021). Newcastle disease is caused by Newcastle disease virus (NDV), which is known as avian paramyxovirus 1 (APMV-1) or avian orthoavulavirus 1 that can induce an intracerebral pathogenicity index (ICPI) of 0.7 or greater in day-old chicks (Gallus gallus) or has multiple basic amino acids (at least three arginine or lysine residues between residues 113 and 116) at the F2 protein C-terminus and phenylalanine at residue 117 that is the N-terminus of the F1 protein (Swayne and King, 2003; Youn et al., 2004; OIE 2018). Viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic or respiratory and asymptomatic pathotypes were identified according to the clinical signs seen in NDV infected chickens, from which velogenicpathotype is the most virulent (Alexander and Senne, 2008; OIE 2018; Amoia et al., 2021; Dzogbema et al., 2021).

Newcastle Disease virus that belongs to genus Avulavirus, family Paramyxoviridae is an enveloped virus with negative sense single-stranded non-segmented RNA genome (Lamb and Parks, 2007; Dimitrov et al., 2019; Dzogbema et al., 2021). The viral genome is made up from six structural genes at the order of 3'-NP-P-M-F-HN-L-5' from which six structural polypeptides are coded (Chambers et al., 1986; Czeglédi et al., 2006 and Lamb and Parks, 2007). Both fusion (F) and Haemagglutinin–neuraminidase (HN) proteins are surface proteins and playing major roles in infection and antigenicity of NDV (de Leeuw, 2005; Kim et al., 2011). Fusion protein is cleaved by host cell proteases into N-terminal F2 (12.5 kDa) and C-terminal F1 (55 kDa) subunits that linked by a disulfide (-S-S-) bridge (Nagai et al., 1989; Bossart et al., 2009). Viral fusion with the host cell membrane is initiated by a fusion peptide (FP) which is formed by a stretch of hydrophobic amino acids that is located at the N terminus of the F1 subunit (Samal et al., 2012 and Selim et al., 2018).

Based on the analysis of complete genome sequences of NDV, three different genome sizes were identified (15,186; 15,192 and 15,198 nucleotides) upon which NDV strains were divided into two classes (class I and class II) (Czeglédi et al., 2006). Class I NDV group encompasses a single genotype that subdivided into 3 sub-genotypes meanwhile class II NDV is divided into 21 genotypes (I–XXI) that subdivided into several sub-genotypes. Genotype VII is subdivided into 3 sub-genotypes; VII.1.1 (formerly includes sub-genotypes VIIb, VIId, VIIe, VIIj and VIIl), sub-genotypeVII.1.2 (formerly known as sub-genotype VIIf) and sub-genotype VII.2 (formerly includes sub-genotypes VIIa, VIIh, VIIi and VIIk) (Dimitrov et al., 2019). Sub-genotype VII.1.1 is prevalent at commercial chicken flocks and wild birds in Egypt (Nagy et al., 2020; AbdElfatah et al., 2021). Due to frequent occurrence of ND outbreaks in Egypt although application of a lot of vaccination programs at many poultry farms, it was important to determine the prevalent viral strains and to determine its genetic divergence from the previously reported and vaccine strains in order to control the disease properly.

1. **Materials and methods:**
   1. **Sample collection:**

Fifty broiler farms in six Egyptian governorates (Behera, Kafr El-sheikh, Sharkia, Alexandria, Dakahlia and Damietta) were found to suffer from diarrhea, respiratory and/or nervous manifestations. Trachea, lung, spleen, proventriculus, duodenum, cecal tonsils and brain were collected from these farms during the period 2020–2022.

* 1. **Viral isolation:**

Samples from each farm were minced, ground and suspended in 10% sterile phosphate-buffered saline (PBS, pH 7.0) that was supplemented with penicillin (1,000 IU/ml) and streptomycin (100 µg/ml). The sample suspension was centrifuged at 448 g for 10 min at 4ºC and filtered through a 0.2 μm filter (Nagy et al., 2020). Each sample was inoculated into allantoic cavity of 9-day-old SPF embryonated chicken egg and incubated at 37°C for 4 days. Allantoic fluids were harvested and stored at – 80 °C till virus identification by RT-PCR.

* 1. **Detection of viral RNA by real time reverse transcriptase−PCR (rRT−PCR):**

Viral RNA was extracted from each sample using QiaAmp viral RNA mini kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions.

The extracted RNA was detected using Quantitect probe RT-PCR kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's instructions. Briefly, for each sample, a reaction mix is composed of 12.5 µl of 2x Quantitect probe RT-PCR buffer with 4.5 µl of RNase - free water and 0.125 µl of Quantitect probe RT enzyme. To each reaction mix the following components were added: 2.25 µl from each primer (10 pmol/µl) and 1µl from probe (5 pmol/ µl). Two sets of primers and probes were used, one directed to matrix gene and the other to fusion gene (table 1).

* 1. **Identification and genetic characterization of viral RNA by RT-PCR:**

Fusion gene was partially amplifiedusing Qiagen OneStep RT-PCR Kit (Qiagen, USA) with the primers set in table 1. The reaction was done in a thermocycler (Biometra, Germany) according to the manufacturer's instructions. AllPCR products were visualized with 100 bp DNA Ladder (Thermo Scientific™ GeneRuler™) on 1.5% agarose gel containing ethidium bromide. DNA fragments were purified using QIAquick Gel Extraction Kit Protocol (Qiagen Inc. Valencia, CA, USA) according to the manufacturer’s instructions. Sequencing reaction was performed using (Big dye Terminator V3.1 cycle) sequencing kit (Perkin-Elmer, Foster city, CA). Sequence reaction was purified using (Centrisep) spin column. Sequencing was performed in an automated sequencer, ABI (Applied Biosystems 3130 genetic analyzer, USA).

**Table 1. Primers and probes used in this study**

|  |  |  |  |
| --- | --- | --- | --- |
| **Targeted gene** | **Primers’ sequences (5′→3′)** | **Probes’ sequences (5′→3′)** | **References** |
| **Matrix gene** | **M+4100:**  **AGTGATGTGCTCGGACCTTC**  **M-4220 :**  **CCTGAGGAGAGGCATTTGCTA** | **M+4169:**  **[FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]** | **Wise et al., 2004** |
| **Fusion gene**  **(detection)** | **F+4839:**  **TCCGGAGGATACAAGGGTCT**  **F-4939:**  **AGCTGTTGCAACCCCAAG** | **F+4894 (VFP-1):**  **[FAM]AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]** |
| **Fusion gene**  **(sequencing)** | **NDV-F330:**  **GGAAGGAGACAAAAACGTTTTATAGG**  **NDV-R700:**  **TCAGCTGAGTTAATGCAGGGGAGG** |  | **Selim et al., 2018** |

* 1. **Phylogenetic analysis:**

Basic local alignment search tool (BLAST) was applied within GenBank database to determine the closely related strains. Nucleotide alignment was done using CLUSTALW tool of BioEdit software (version 7.2.5). Maximum composite likelihood method of MEGA11 software was used to determine pairwise distance in relation to other NDV strains. Neighbor-joining (NJ) tree was constructed using maximum composite likelihood method with 1000 bootstrap replications to determine genotypes and sub-genotypes of the isolated strains (Tamura et al., 2021).

1. **Results and discussion:**

Newcastle disease (ND) is considered to be one of the world widely distributed and economically significant avian diseases (Abd Elfatah et al., 2021). In Egypt, many vaccination programs were adopted to control the disease. However, many outbreaks are frequently reported due to inadequate vaccination practices, emergence of new virulent NDV strains and poor biosecurity measures (Nagy et al., 2020). Therefore, continuous monitoring of the viral genetic changes and determination of its divergence from the vaccine strains are important for proper disease control. During this study an effort was done to identify NDV in clinical samples that were collected from broilers at 50 poultry farms in six Egyptian governorates during the period 2020–2022. Sampleswere subjected to real-time RT-PCR targeting Matrix (M) gene which is a basic protein and has several conserved hydrophobic regions (Bellini et al., 1986 and Wise et al. 2004). Positive samples were isolated in embryonated chicken eggs, and NDV was subsequently identified in allantoic fluids by real-time RT-PCR targeting fusion (F) gene of Velogenic strain (Wise et al. 2004). Moreover, partial sequencing was performed to the fusion gene including cleavage site and the N terminus of the F1 subunit that is essential in initiation of viral fusion with the host cell membrane (Samal et al., 2012). Nineteen NDV isolates namely (HS1NDV−HS19NDV) were identified and deposited on GeneBank under the accession nos. (OP588159−OP588177). These isolates were identified in vaccinated broiler farms except the isolates HS5NDV and HS17NDV that were identified in nonvaccinated farms (table 2). Identification of NDV in vaccinated farms can be returned to the fact that vaccination can reduce or prevent clinical disease; nevertheless the virus may still circulate in vaccinated ﬂocks (Perozo et al., 2012; Rehmani et al., 2015; Dimitrov et al., 2017; Goraichuk et al., 2020). The nucleotide distances between the identified isolates ranged from 0%−5.5% and the amino acid distances ranged from 0%−3.6% (table 3). Amino acid distances of 0%−1.8% were found between the strains that isolated during 2020. Meanwhile, amino acid distances of 0%−3.6% were found between the strains that isolated during 2021. The same amino acid distances were found between the strains that isolated during 2022 (table 3). Strain HS2NDV that was identified in Alexandria during 2020, was genetically identical to HS5NDV that was identified in Dakahlia during the same year. However, nucleotide distances of 2.4%−3.9% were found between the strains that were identified from the two localities during 2021. Moreover, nucleotide distance of 4.3% was found between the strains that were identified from the two localities during 2022. In addition, the highest nucleotide distance (5.5%) that was reported between the all isolates and was previously set as one of the required criteria for naming a new sub-genotype (Dimitrov, et al., 2019), was found between the isolate from Alexandria (HS14NDV) during 2021 and from Dakahlia (HS17NDV) during 2022 (table 3). These findings can highlights the different genetic evolution of the viruses that isolated from different localities although their previous genetic identical character.

Former sub-genotypes VII b, VII d, VII e, VII j and VII l did not fulfill the distance required (above 5%) to form separate sub-genotypes according to Dimitrov, et al. and therefore they were merged into a single sub-genotype VII.1.1 (Dimitrov, et al., 2019). Herein, strains HS6NDV and HS9NDV−HS18NDV had distances above 5% from former sub-genotypes VII b, VII d, VII e and VII I and had separate branch in relation to these former sub-genotypes and sub-genotype VII j (table 4 and figure 2). Meanwhile, these strains altogether with the other identified ones had distances less than 5% from former sub-genotype VII j (table 4). Thus, all the strains that were identified during this study can be assigned under genotype VII and sub-genotype VII.1.1.

Fusion peptide (FP) is a stretch of hydrophobic amino acids that located at the N terminus of the F1 subunit and initiates the fusion process via interaction with the host cell membrane (Samal et al., 2012; Selim et al., 2018). In relation to vaccine strains, 5 strains that were identified during the current study had unique amino acid substitutions at the FP region. These substitutions were found at 3 residues (121, 122 and 123) (figure 3). F1 subunit of F protein has two heptad repeat (HR) motifs; one of which is HR1 that is adjacent to the fusion peptide. Conserved six-helix bundles are formed via assembly of these heptad repeats and this assembly is tightly coupled to the membrane fusion (Samal et al., 2012; Selim et al., 2018). Strain HS3NDV has one amino acid substitution Asn145Lys at the HR1. Additionally, unique amino acid substitutions were found at residues 215, 220 and 222 in relation to vaccine strains (figure 3). All the isolates found to be velogenic according to the amino acid sequences of the cleavage sit that was (GRRQKRF, figure 3) (Swayne and King, 2003; Youn et al., 2004; OIE 2018). The strains that were isolated during 2020, were identical at the amino acid level except HS3NDV, that had two amino acid substitutions (Asn145Lys and Ile215Thr) one of which (Asn) is located at the heptad repeat motif (HR1)( Samal et al., 2012 and Selim et al., 2018).

In conclusion, NDV was identified in broiler farms at six Egyptian governorates along 3 years and the isolates were characterized under genotype VII and sub-genotype VII.1.1. In relation to vaccine strains, amino acid substitutions were found at some isolates in their fusion peptides that are essential in initiation of viral fusion with the host cell membrane. In addition, one amino acid substitution was found at the strain HS3NDV in HR1 which has a role the membrane fusion. This can indicate the frequent genetic mutation of NDV that was distinct from vaccine strains.

**Table 2. Epidemiological data of the isolates**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Strain ID | GenBank accession nos. | Year of collection | Governorate | Vaccination |
| HS1NDV | OP588159 | 2020 | Sharkia | Vaccinated |
| HS2NDV | OP588160 | 2020 | Alexandria | Vaccinated |
| HS3NDV | OP588161 | 2020 | Kafr El-sheikh | Vaccinated |
| HS4NDV | OP528816 | 2020 | Behera | Vaccinated |
| HS5NDV | OP588163 | 2020 | Dakahlia | nonvaccinated |
| HS6NDV | OP588164 | 2021 | Kafr El-sheikh | Vaccinated |
| HS7NDV | OP588165 | 2021 | Alexandria | Vaccinated |
| HS8NDV | OP588166 | 2021 | Behera | Vaccinated |
| HS9NDV | OP588167 | 2021 | Behera | Vaccinated |
| HS10NDV | OP588168 | 2021 | Sharkia | Vaccinated |
| HS11NDV | OP588169 | 2021 | Dakahlia | Vaccinated |
| HS12NDV | OP588170 | 2021 | Dakahlia | Vaccinated |
| HS13NDV | OP588171 | 2021 | Damietta | Vaccinated |
| HS14NDV | OP588172 | 2021 | Alexandria | Vaccinated |
| HS15NDV | OP588173 | 2021 | Behera | Vaccinated |
| HS16NDV | OP588174 | 2022 | Sharkia | Vaccinated |
| HS17NDV | OP588175 | 2022 | Dakahlia | nonvaccinated |
| HS18NDV | OP588176 | 2022 | Kafr El-sheikh | Vaccinated |
| HS19NDV | OP588177 | 2022 | Alexandria | Vaccinated |

**Table 3.** **Nucleotide and Amino acid distances between the identified strains**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Amino acid distances (%) | | | | | | | | | | | | | | | | | | | | |
|  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** |  |
| 1- HS1NDV |  | 0 | 1.8 | 0 | 0 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | **1** |
| 2- HS2NDV | 0 |  | 1.8 | 0 | 0 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | **2** |
| 3- HS3NDV | 2.1 | 2.1 |  | 1.8 | 1.8 | 3.6 | 3.6 | 2.7 | 3.6 | 3.6 | 3.6 | 3.6 | 2.7 | 3.6 | 3.6 | 3.6 | 3.6 | 3.6 | 1.8 | **3** |
| 4- HS4NDV | 2.1 | 2.1 | 4.3 |  | 0 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | **4** |
| 5- HS5NDV | 0 | 0 | 2.1 | 2.1 |  | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | **5** |
| 6- HS6NDV | 2.4 | 2.4 | 4.6 | 3.3 | 2.4 |  | 0.9 | 0.9 | 1.8 | 3.6 | 3.6 | 3.6 | 2.7 | 3.6 | 3.6 | 3.6 | 1.8 | 3.6 | 3.6 | **6** |
| 7- HS7NDV | 2.1 | 2.1 | 4.3 | 3 | 2.1 | 0.6 |  | 0.9 | 1.8 | 3.6 | 3.6 | 3.6 | 2.7 | 3.6 | 3.6 | 3.6 | 1.8 | 3.6 | 3.6 | **7** |
| 8- HS8NDV | 1.8 | 1.8 | 4 | 2.7 | 1.8 | 1.2 | 0.9 |  | 0.9 | 2.7 | 2.7 | 2.7 | 1.8 | 2.7 | 2.7 | 2.7 | 1.8 | 2.7 | 2.7 | **8** |
| 9- HS9NDV | 2.4 | 2.4 | 4.6 | 3.3 | 2.4 | 1.5 | 1.5 | 0.6 |  | 3.6 | 3.6 | 3.6 | 2.7 | 3.6 | 3.6 | 3.6 | 2.7 | 3.6 | 3.6 | **9** |
| 10- HS10NDV | 3.3 | 3.3 | 4.9 | 4.9 | 3.3 | 4.6 | 4.3 | 3.9 | 4.6 |  | 0 | 0 | 0.9 | 0 | 0 | 0.9 | 3.6 | 0 | 1.8 | **10** |
| 11- HS11NDV | 2.7 | 2.7 | 4.9 | 43 | 2.7 | 3.9 | 3.6 | 3.3 | 3.9 | 0.6 |  | 0 | 0.9 | 0 | 0 | 0.9 | 3.6 | 0 | 1.8 | **11** |
| 12- HS12NDV | 3 | 3 | 5.2 | 4.6 | 3 | 4.3 | 3.9 | 3.6 | 4.3 | 0.3 | 0.3 |  | 0.9 | 0 | 0 | 0.9 | 3.6 | 0 | 1.8 | **12** |
| 13- HS13NDV | 2.4 | 2.4 | 4.6 | 4 | 2.4 | 3.6 | 3.3 | 3 | 3.6 | 0.9 | 0.3 | 0.6 |  | 0.9 | 0.9 | 0.9 | 2.7 | 0.9 | 0.9 | **13** |
| 14- HS14NDV | 2.7 | 2.7 | 4.6 | 3.6 | 2.7 | 2.7 | 2.4 | 2.1 | 2.7 | 2.4 | 2.4 | 2.4 | 2.7 |  | 0 | 0.9 | 3.6 | 0 | 1.8 | **14** |
| 15- HS15NDV | 2.4 | 2.4 | 4.6 | 3.3 | 2.4 | 2.4 | 2.1 | 1.8 | 2.4 | 2.4 | 2.1 | 2.1 | 2.4 | 0.3 |  | 0.9 | 3.6 | 0 | 1.8 | **15** |
| 16- HS16NDV | 3.3 | 3.3 | 5.2 | 4.9 | 3.3 | 5.2 | 4.9 | 4.6 | 5.2 | 1.8 | 2.1 | 1.8 | 2.1 | 3 | 3.3 |  | 3.6 | 0.9 | 1.8 | **16** |
| 17- HS17NDV | 3.6 | 3.6 | 5.2 | 5.2 | 3.6 | 3.7 | 4.3 | 3.7 | 4 | 3.9 | 3.9 | 4.3 | 3.6 | 5.5 | 5.5 | 3 |  | 3.6 | 3.6 | **17** |
| 18- HS18NDV | 2.7 | 2.7 | 4.9 | 4.3 | 2.7 | 4.6 | 4.3 | 3.9 | 4.6 | 1.2 | 1.2 | 0.9 | 1.5 | 2.7 | 2.4 | 0.9 | 3.3 |  | 1.8 | **18** |
| 19- HS19NDV | 2.4 | 2.4 | 4 | 3.3 | 2.4 | 4.3 | 3.9 | 3.6 | 4.3 | 3.3 | 2.7 | 3 | 2.4 | 3.3 | 3 | 2.7 | 4.3 | 2.1 |  | **19** |
| Nucleotide distances (%) | | | | | | | | | | | | | | | | | | | |  |

**Table 4. Nucleotide divergence (%) between the identified isolates and the other strains that representing different sub-genotypes of genotype VII**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Strain ID** | **VII.1.1** | | | | | **VII.1.2** | **VII.2** | | | |
| **VII b** | **VII d** | **VII e** | **VII j** | **VII l** | **VII f** | **VII a** | **VII h** | **VII i** | **VII k** |
| **KJ450977** | **EU583503** | **LC650548** | **KC542905** | **KX268351** | **DQ858357** | **JN986837** | **MF622047** | **KU862293** | **KY747479** |
| **HS1NDV** | **3.4** | **3.3** | **3.7** | **1.8** | **5.9** | **4.9** | **6.6** | **11.3** | **9.4** | **11** |
| **HS2NDV** | **3.4** | **3.3** | **3.7** | **1.8** | **5.9** | **4.9** | **6.6** | **11.3** | **9.4** | **11** |
| **HS3NDV** | **3** | **2.4** | **2.7** | **2.7** | **5.6** | **4** | **5.6** | **11** | **8.3** | **10** |
| **HS4NDV** | **4.3** | **4.3** | **4** | **2.7** | **5.6** | **5.3** | **6.9** | **10.3** | **9.7** | **10.7** |
| **HS5NDV** | **3.4** | **3.3** | **3.7** | **1.8** | **5.9** | **4.9** | **6.6** | **11.3** | **9.4** | **11** |
| **HS6NDV** | **5.2** | **5.9** | **5.6** | **3.6** | **7.9** | **6.9** | **7.9** | **13.5** | **11.4** | **12.4** |
| **HS7NDV** | **4.9** | **5.6** | **5.2** | **3.3** | **7.6** | **6.5** | **7.5** | **13.1** | **11.1** | **12** |
| **HS8NDV** | **4.6** | **5.2** | **4.6** | **3** | **7.2** | **5.9** | **6.9** | **12.4** | **10.4** | **11.7** |
| **HS9NDV** | **5.2** | **5.9** | **5.2** | **3.6** | **7.9** | **6.5** | **7.5** | **13.1** | **11.1** | **12.4** |
| **HS10NDV** | **5.6** | **6.2** | **6.4** | **4** | **7.6** | **7.2** | **7.5** | **13.8** | **11.1** | **12** |
| **HS11NDV** | **5.6** | **6.2** | **5.9** | **4** | **7.6** | **7.2** | **7.5** | **13.8** | **11.1** | **12** |
| **HS12NDV** | **5.9** | **6.5** | **6.2** | **4.3** | **7.9** | **7.6** | **7.9** | **14.2** | **11.4** | **12.4** |
| **HS13NDV** | **5.3** | **7.1** | **5.6** | **3.7** | **7.2** | **6.9** | **7.2** | **13.5** | **10.7** | **11.7** |
| **HS14NDV** | **5.2** | **5.9** | **5.5** | **3.6** | **7.9** | **6.8** | **7.8** | **13.4** | **11.4** | **12.3** |
| **HS15NDV** | **5.2** | **5.9** | **5.5** | **3.6** | **7.9** | **6.9** | **7.9** | **13.4** | **11.4** | **12.4** |
| **HS16NDV** | **5.9** | **6.5** | **6.2** | **4.3** | **7.9** | **7.5** | **9.2** | **14.2** | **11.4** | **13.9** |
| **HS17NDV** | **5.9** | **6.5** | **5.9** | **4.3** | **7.9** | **7.2** | **8.9** | **13.8** | **11.1** | **13.9** |
| **HS18NDV** | **5.6** | **6.2** | **5.9** | **4** | **7.6** | **7.2** | **8.9** | **13.8** | **11.1** | **13.5** |
| **HS19NDV** | **4.6** | **5.2** | **4.9** | **3** | **6.6** | **6.2** | **7.9** | **12.7** | **10** | **12.8** |

Accession numbers indicate the following strains; KJ450977 (Jiangxi 07), EU583503 (Hebei), LC650548 (Ibaraki-1), KC542905 (Liaoning 01), KX268351 (Behshahr), DQ858357 (Jiangsu YG), JN986837 (152608 ancestral), MF622047 (RBWW 3), KU862293 (Karachi AW 1) and KY747479 (5620).

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**Figure 1. Neighbor joining tree demonstrates genotyping of the identified isolates**



**Figure 2. Sub-genotyping of the isolated strains using maximum composite likelihood with 1000 bootstrap replications.**

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**Figure 3. Amino acid substitutions between the identified strains and vaccine strains. Red rectangular indicates cleavage site. Green rectangulars indicate the unique amino acid residues of NDV isolated during this study.**

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