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Molecular Characterization of Two Selected Pigeon Paramyxovirus-1 Isolates Reveals Two Different Cleavage Site Amino Acid Motifs

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Key words:
Pigeon Paramyxovirus-1, F gene, Phylogenetic analysis, Amino acid motifs

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ABSTRACT

The aim of this study was the isolation, identification and molecular characterization of Avian (Pigeon) Paramyxovirus-1 (APMV-1) isolated from clinically affected pigeons suspected to be infected with PPMV-1 in Egypt between 2016 and 2017. Twenty-five field samples were collected and inoculated into the allantoic cavity of ECE. Allantoic fluids were tested for haemagglutinating (HA) activity followed by haemagglutination inhibition (HI) test for virus identification. Molecular confirmation was done by reverse transcription polymerase chain reaction (RT-PCR) using primers specific to the fusion (F) gene. Results revealed 12 out of 25 positive samples. Two samples were selected for sequencing and phylogenetic analysis which revealed that two different amino acid motifs were found at the cleavage site of F protein: [112] KRQKRF[117] (associated with virulent strains) and [112]GRQGRL[117] (associated with lentogenic strains).To our knowledge, this is the first reported PPMV-1 isolates that possess the sequences of [112]GRQGRL[117] within the F0 protein.

1. INTRODUCTION:

Avian Paramyxovirus-1 (APMV-1) is the causative agent of the Newcastle disease (ND) and is known in pigeons as the pigeon paramyxovirus type 1 (PPMV-1) which is one of the most important infectious pigeon diseases worldwide. It is a host variant of the NDV of chickens and there is a close antigenic relationship between both viruses. They are classified under family Paramyxoviridae and within the same serotype (Avian Paramyxovirus type 1) (Akhtar *et al*., 2016). It has been proposed that APMV-1 of pigeon was emerged as a result of predominant transmission of the virus from chickens to pigeons (Mayahi et al., 2017).Outbreaks of PPMV-1 are frequently occurring in Egypt remaining a serious threat to the commercial poultry population due to the huge economic impacts (Mansour et al., 2017).

Although APMV-1 is mainly pathogenic to pigeons, but recently some APMV-1of pigeon have been reported to be highly pathogenic for chicken after propagation in ECE indicating the high risk to cause ND outbreaks (Akhtar *et al*., 2016). Previous studies concluded that PPMV-1 strains are firstly transmitted from chicken to pigeon .Then these PPMV-1 isolates appear to be more adapted for pigeons and gradually lose their virulence for chickens (Suarez, 2013). Pigeon-origin APMV-1 is highly contagious and can lead to serious outbreaks in poultry (He *et al*., 2018).

Signs of infection with APMV-1 generally resemble the symptoms of the neurotropic velogenic form of ND (Pestka *et al*., 2014). In pigeons and doves, clinical manifestations include nervous signs such as torticollis, incoordination, and tremors, paralysis of wings and/or legs and difficulties of food intake, in

addition to greenish watery diarrhea (Marlier and Vindevogel, 2006).

The first ND outbreaks were reported in 1926 in Indonesia and England (Doyle, 1927) while APMV-1 as an antigenic variant of NDV originated from the Middle-East (ME) in 1978 and was known as (Iraq78 strain) (Marina *et al*., 2007). APMV-1 was first diagnosed in Egypt as a neurotropic form of ND among pigeon (El-Dahaby and Sokkar, 1976) but was not identified as APMV-1 of pigeon except in 1981 (Eskelund, 1986).

APMV-1 belonging to class I viruses contain a one genotype while class II APMV-1 can be further classified into eighteen genotypes (I - XVIII) (Diel et al., 2012). Most pigeon-origin virulent APMV-1 isolates are classified under genotype VI of class II, which can be further sub-divided into VIa - VIg. The VIa strains were isolated in the 1960s from the Middle East and rapidly spread to the African and Asian countries (Qiu *et al*., 2017).

Paramyxoviruses have two envelope glycoprotein spikes on their outer envelope: the fusion protein (F) spike and the haemagglutinin-neuraminidase (HN) spike which are critical in the viral pathogenesis (MacLachlan *et al*., 2011). During the APMV1 multiplication cycle, the F protein is originally generated as precursor F0; which must be cleaved into F1 and F2 polypeptides which is critical in mediating fusion of the viral envelope with cell membranes (Gowthaman *et al.,* 2016).

The APMV-1 can be characterized by means of phylogenic analysis of the fusion (F) gene (Molini et al., 2018), intracerebral pathogenicity index (ICPI), intravenous pathogenicity index and mean death time (MDT) into velogenic, mesogenic and lentogenic strains (Mayahi et al., 2017).

The amino acid sequence of the F protein cleavage sites of the velogenic and mesogenic strains is 112(R/K) RQ(R/K)RF117 while the lentogenic strains have a monobasic amino acid motif at the same site, 112 (G/E)(K/R)Q(G/E)RL117 and so the F protein of these strains is cleaved only by trypsin-like enzymes, which are present only in the respiratory tracts. Conversely, the F protein of the velogenic strains such are cleaved by ubiquitous and diverse host protease (Chen et al., 2013). The World Organization for Animal Health defined the velogenic APMV-1 as a virus with at least three multiple basic amino acids (arginine or lysine) residues at the C-terminus (between residues 113 and 116) and phenylalanine at residue 117. (Qiu et al., 2017)

Although the pigeon-origin APMV-1 are the cause of the many NDV outbreaks of pigeons and occasionally poultry, but thorough characterization of the genomes of these APMV-1 is of limited study (He et al., 2018) Therefore, molecular characterization of the isolates is essential to come to a complete and accurate diagnosis of the disease. In this study we report the isolation and characterization of APMV-1 strains isolated from pigeons in Egypt between 2016 and 2017 with sequencing and phylogenetic analysis of the partial F protein gene fragment containing the cleavage site of two selected isolates

2. MATERIALS AND METHODS:

2.1. Field samples

A total number of 25 freshly dead tissue samples (brain, lung, trachea, liver, spleen and kidney) were collected from field cases of pigeons suffering from nervous manifestations (tremors and torticollis), greenish diarrhea and high mortality rate with no previous history of vaccination. These samples were collected during the period from August 2016 till January 2017. Collected organs were cut into fine pieces, grinded as a pool, homogenized and suspended in PBS to make 20% W/V suspension which was subjected to 3 cycles of freezing and thawing then centrifuged at 3000 rpm/15 min. Tissue supernatants were collected and stored at -80º C till being used in virus isolation and identification.

Table (1): Oligonucleotide primers used for amplification of F gene (**Leeuw** et al., 2005).

Primer	Oligonucleotide sequence	Position	Length of amplified fragment
Forward	5'-GCAGCTGCACGGATTGTGGT-3'	(nt 158-177)	356 **bp**
Reverse	5'-TCTTTGAGCAGGAGGATGTTG-3'	(nt 513-493)	

2.2. Virus isolation and identification

Prepared samples were antibiotic and antimycotic treated then inoculated into 10-days-old specific pathogen free (SPF) ECE via allantoic route for three successive blind passages [OIE, 2012]. Inoculated eggs were incubated at 37 ºC for 96 h and candled daily for embryo mortality. Death recorded within first 24 h was excluded as non-specific death. The eggs were chilled at 4°C overnight and allantoic fluids were collected and centrifuged for testing the HA activity. The collected allantoic fluids were subjected to a rapid haemagglutination (RHA) test followed by microtitre plate HA and HI tests according to (Omar *et al*., 2014). Hyperimmune anti-APMV-1 serum, prepared in rabbits by repeated injection with an APMV-1 inactivated vaccine (VSVRI, Abassia, Cairo) was used in HI test. Negative fluids were inoculated for three blind passages into further batches of eggs. Commercially available Hitchner and Lasota vaccines were also used in HI test for compatibility evaluation and comparison.

2.3. Molecular identification

Allantoic fluids showing positive HA activity were submitted to RNA extraction by using QIAamp viral RNA mini Kit following the manufacturer's protocol (Qiagen, USA). RT-PCR was performed using Qiagen One Step RT-PCR Kit according to manufacturer's protocol. Primers in (Table 1) were used to amplify a 356 bp fragment of F protein gene containing the cleavage site. PCR thermal cycle conditions were as follow: reverse transcription at 50 ºC for 30 min then an initial denaturation at 95 ºC / 15 min followed by 40 cycles of (denaturation at 94 ºC / 30 sec, annealing at 58 ºC / 30 sec, extension at 72 ºC / 45 sec) then final extension at 72 ºC / 10 min. A negative control consisting of primers and PCR grade water was also included .The resulting RT-PCR products were analyzed by agarose gel electrophoresis and visualized under the UV light.

2.4. Sequencing and phylogenetic analysis

For gene sequencing, the target bands were purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) followed by sequencing using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster city, CA) and Applied Biosystems 3130 genetic analyzer (ABI, United States). Sequence similarities of two selected isolates were compared with previously published APMV-1 vaccines and reference strains available in the public database (NCBI, United States) and analyzed using the CLUSTAL W Multiple Sequence Alignment Program, version 1.83 of MegAlign module of Lasergene DNAStar software. Amino acids phylogenetic tree through a bootstrap of 1000 trials was drawn for the sequenced isolates along with other vaccine and reference strains available in the GenBank database using MEGA version 6 (Tamura *et al.,* 2013).

3. RESULTS

3.1. Clinical signs and gross pathology.

The clinical examination of infected pigeons revealed general signs of illness, anorexia, nervous signs and white greenish diarrhea. The nervous signs include incoordination, tremors, head deviation (torticollis) and opisthotonus position .Affected pigeons were unable to eat or fly. Course of infection was variable ranging from 1-14 days. Morbidity rates were generally high while mortality rates ranged from 10% to 50% in some pigeon lofts.

PM examination of affected pigeons revealed enteritis with greenish content in the proventriculus and along the intestinal tract. Some pigeons frequently showed congested pectoral muscles, lung, liver and/or spleen (splenomegally in some pigeons) and cloudness of the air sacs. Gastrointestinal cestodes were present in the intestinal tract of some examined affected pigeons.

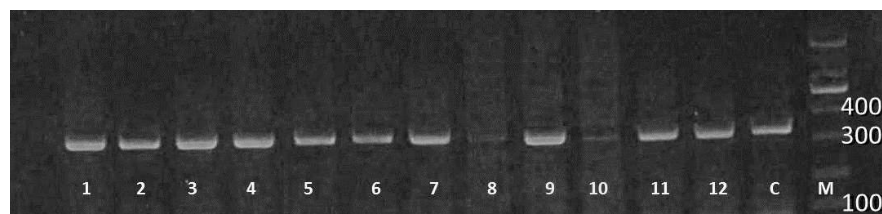

Fig (1) Amplification of F protein gene fragment (356 bp) by RT-PCR. M = Marker (100bp), C = Positive Control, 1-12 are PPMV-1field isolates.

3.2. Virus isolation and identification.

No embryo mortalities were detected during the first 4 days of inoculation in the first egg passage. Embryo mortalities detected in the 2nd, 3rd and 4th passages were during the 3rd and 4th days post inoculation. No gross changes were detected in the dead embryos. Only slight congestion of chorioallantoic membrane (CAM) was observed in some ECE. Allantoic fluids from both freshly dead and live embryos were harvested for testing of HA activity. Rapid slide HA test was performed for all harvested allantoic fluids and revealed that 12 out of 25 samples were positive for HA .Two out of the 12 samples were positive for HA after 1st passage, 3 samples after 2nd passage and the other 7 samples were HA positive only after 3rd and 4th passages. Microtiter plate HA test was then performed for detection of the HA titer of positive samples which ranged from 2^6 to 2^{11}. Haemagglutinating activity of these samples was inhibited by PPMV specific hyperimmune serum in HI test, which is confirmatory for paramyxoviruses. HI titer of hyperimmune serum was variable for different samples and ranged from 2^8 to more than 2^{12}. HI titers of the antiserum were 2^{12} and 2^9 for Hitchner and Lasota vaccines respectively.

3.3. Molecular identification.

All 12 HA and HI positive allantoic fluids were RT-PCR positive and the partial fragment of (F) protein gene (356 bp) was successfully amplified giving the specific bands (Fig 1).

3.4. Sequencing and genetic analysis.

The partial F gene sequences of the PPMV-1 of two selected isolates (Pigeon/Egypt/KFS/2017/2) and (Pigeon/Egypt/KFS/2017/5) were deposited in the GeneBank under accession numbers (MF375924) and (MF614961) respectively. Compared to reference and vaccinal strains available in the GenBank database, the identity and divergence of the isolate (Pigeon/Egypt/KFS/2017/2) ranged between 80.2–98% and 2– 23% on amino acid levels respectively and for the other selected isolate (Pigeon/Egypt/KFS/2017/5) was 81.2-93.1% for identity and 7.3-21.7% for divergence (Fig 4). The amino acid sequence of the F0 protein cleavage site motif (residues 112 to 117) of F-gene of the selected strains (Pigeon/Egypt/KFS/2017/2) and (Pigeon/Egypt/KFS/2017/5) was ^{112}KRQKRF^{117} and ^{112}GRQGRL^{117} respectively (Fig 3). The phylogenetic analysis of partial sequences of the selected isolates F gene showed that the isolate (Pigeon/Egypt/KFS/2017/2) belongs to class II genotype VIb.2. While the other isolate (Pigeon/Egypt/KFS/2017/5) was distinct from other isolates and formed a single clade closer to the vaccinal strains (Fig 5).

4. DISCUSSION

Pigeon production is a main part of many rural farmers' life in Egypt as it is a source of low cost white meat if compared with other poultry species (Omar *et al.*, 2014). Avian (Pigeon) paramyxovirus type 1 viruses are antigenic variants of NDV that infect pigeons since the late 1970s. Outbreaks of ND in pigeons are still being reported across the world, including Egypt. In spite of control measures, specially, vaccination, APMV-1 infection remains enzootic in pigeons in many countries leading to economic loss (Guo *et al.*, 2013; Mase and Kanehira, 2015; Wang *et al.*, 2015). In this study, 25 tissue samples were collected from field cases of pigeons for isolation and identification of APMV-1.

These pigeons suffered from neurologic signs with variable mortalities (10–50%) which suggest APMV infection. High mortality rates observed in some pigeon lofts may be attributed to concurrent bacterial and/or parasitic infections (Marlier and Vindevogel, 2006). The first step in proper diagnosis of APMV-1 is virus isolation [24] which was performed via allantoic route of 10 –days- old ECE (OIE, 2012).Infected allantoic fluids were tested for HA activity as this property of the virus and its inhibition by specific antisera have proven to be powerful tools in the diagnosis of the disease (Burnet, 1943). Haemagglutination test revealed that 12 out of 25 samples were positive for HA. Since all serotypes of APMV and avian influenza viruses (AI) have HA activity, therefore, HI test with polyclonal antisera of rabbits immunized with APMV-1 had been conducted to the HA positive allantoic fluids (Śmietanka *et al.*, 2006) which revealed that all tested allantoic fluids were HI positive which confirmed the PPMV-1 infection. HI titers of polyclonal antiserum were variable with the tested samples and ranged from 2^8 to 2^{12} which indicated that the samples with higher titers have more compatibility with the vaccinal APMV-1 from which antiserum had been prepared.

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Majority                                   AACATATACACCTCATCCCAGACAGGGTCAATCATAGTCAAGTTCCTCCCGAATCTGCCCAAGGATAAGGAGGCATGTGC
                                           ---------+---------+---------+---------+---------+---------+---------+---------+
                                                   10        20        30        40        50        60        70        80
                                           ---------+---------+---------+---------+---------+---------+---------+---------+
NDV-Chicken/China/SDWF07/2011              AATGTATACACCTCGTCTCAGACAGGGTCAATCATAGTCAAGTTGCTCCCGAATATGCCCAGAGATAAGGAGGCATGTGC    80
NDV-H1 Fusion protein                      ..CA..........A..C........A...........T...C.C.........C......AG.................    80
NDV-LASOTA-II-fusion                       ..CA..........A..C........A...........T...C.C.........C......AG.................    80
NDV- isolate Hitchner fusion genes         ..CA..........A..C........A...........T...C.C.........C......AG.................    80
NDV-clone 30                               ..CA..........A..C........A...........T...C.C.........C......AG.................    80
NDV--VG/GA                                 ..CA..........A..C........A...........T...C.C.........C......AG.................    80
vectormune NDV Strain                      ..CA..........A.....................A.......A.....A..........AG......A.....G....    80
NDV-chicken/N. Ireland/Ulster/67           .CCA.T.C......A.............................A.....A...T......A.......A.....G.T..    80
NDV-FU13-EGYPT-NLQP-2014                   ................................................................................    80
pigeon/Egypt/Sharkia-2/2013 fusion         ...A................................A.............A..........AG............G....    80
Newcastle disease virus strain NDV V4      ..CA..........A.....................A.......A.....A..........AG......A.....G....    80
Newcastle disease virus VG/GA clone 5      ..CA..........A..C........A...........T...C.C.........C......AG.................    80
NDV-avenu                                  ..CA..........A.....................A.......A.....A..........AG......A.....G....    80
Newcastle disease virus B1                 ..CA..........A..C........A...........T...C.C.........C......AG.................    80
Newcastle disease virus strain B1 Takaa    ..CA..........A..C........A...........T...C.C.........C......AG.................    80
Newcastle disease virus strain Musktesw    ..CA..........A..C..........................A.....A..........AG...C..A..........    80
VNDV/CK/EG/VIR-SHABS/2014                  ................................................................................    80
Pigeon/Egypt/KFS/2017/2                    ...A..........A.....................A.............A..........AG............G....    80
Pigeon/Egypt/KFS/2017/5                    ..CA..........A..C........C...........T..TC.CG.......GC......AG............T.G..    80

Majority                                   AAAAGCCCCCTTGGATGCATACAACAGGACATTGACTACTTTGCTCACCCCCCTTGGTGACTCTATCCGTAGGATACAAG
                                           ---------+---------+---------+---------+---------+---------+---------+---------+
                                                   90       100       110       120       130       140       150       160
                                           ---------+---------+---------+---------+---------+---------+---------+---------+
NDV-Chicken/China/SDWF07/2011              AAGAGCCCCACTGGAGGCATATAACAGAACACTGACTACTCTGCTCACTCCTCTTGGTGACTCCATCCGCAAGATCCAAG   160
NDV-H1 Fusion protien                      G.A.A....CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160
NDV-LASOTA-II-fusion                       G.A......CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160
NDV- isolate Hitchner fusion genes         G.A......CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160
NDV-clone 30                               G.A......CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160
NDV--VG/GA                                 G.A......CT....T...........G...T....C...T.......C..C...........T.....T.GA..A....   160
vectormune NDV Strain                      ..A.......T....A.....C.....G...T........T.......C..C........T..T.....T.G...A....   160
NDV-chicken/N. Ireland/Ulster/67           ..A......G........G.TC.....G...T........TG......C..C........T..T.....T.G...A....   160
NDV-FU13-EGYPT-NLQP-2014                   ..A.......T.....................................................................   160
pigeon/Egypt/Sharkia-2/2013 fusion         .........GT.A..A.....C................G.T.A....AC..C........T..........G...A....   160
Newcastle disease virus strain NDV V4      ..A......GT..........C.....G...T........T.......C..C........T..T.....T.G...A....   160
Newcastle disease virus VG/GA clone 5      G.A......CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160
NDV-avenu                                  ..A......GT..........C.....G...T........T.......C..C........T..T.....T.G...A....   160
Newcastle disease virus B1                 G.A......CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160
Newcastle disease virus strain B1 Takaa    G.A......CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160
Newcastle disease virus strain Musktesw    ..A.......T.......T..C.....G............T....T..C..C........T..T.......G...A....   160
VNDV/CK/EG/VIR-SHABS/2014                  ..........T.....................................................................   160
Pigeon/Egypt/KFS/2017/2                    ..A......GT.A..A.....C................G.T.A....AC..C........T..T.......G..C.....   160
Pigeon/Egypt/KFS/2017/5                    G.A......CT....T.....C.....G...T....C...T.......C..C...........T.....T.G...A....   160

Majority                                   AGTCTGTGACTACATCTGGAGGAGGGAGACAGGGGCGCCTTATAGGCGCCATTATTGGCGGTGTAGCTCTTGGGGTTGCA
                                           ---------+---------+---------+---------+---------+---------+---------+---------+
                                                  170       180       190       200       210       220       230       240
                                           ---------+---------+---------+---------+---------+---------+---------+---------+
NDV-Chicken/China/SDWF07/2011              GGTCTGTATCCACGTCCGGAGGAAGGAGACAAAAACGTTTTATAGGTGCTGTTATTGGCAGTGTAGCTCTTGGGGTTGCA   240
NDV-H1 Fusion protien                      A.....GA..T..A..T.....GG.......GGGG..CC.......C..CA........G....G...............   240
NDV-LASOTA-II-fusion                       A.....GA..T..A..T.....GG.......GGGG..CC..........CA........G....G...............   240
NDV- isolate Hitchner fusion genes         A.....GA..T..A..T.....GG.......GGGG..CC.......C..CA........G....G...............   240
NDV-clone 30                               A.....GA..T..A..T.....GG.......GGGG..CC.......C..CA........G....G...............   240
NDV--VG/GA                                 A...A.GA..T..A..T.....GG.......GGGG..CC.......C..CA........G....G...............   240
vectormune NDV Strain                      A.....GA.....A.........G...A...GGG....C.......C..CA....C..TG..........C.........   240
NDV-chicken/N. Ireland/Ulster/67           A.....GA..T..A..T......G...A...GGG...CC.......C..CA....C...G...C......C.........   240
NDV-FU13-EGYPT-NLQP-2014                   .........................................................................A......   240
pigeon/Egypt/Sharkia-2/2013 fusion         .A...........A..A.......A......G..G..C...........CA....C.......................G   240
Newcastle disease virus strain NDV V4      A.....GA...............G...A...GGG....C.......C..CA....C..TG..........C.........   240
Newcastle disease virus VG/GA clone 5      A.....GA..T..A..T.....GG.......GGGG..CC.......C..CA........G....G...............   240
NDV-avenu                                  A.....GA...............G...A...GGG....C.......C..CA....C..TG..........C.........   240
Newcastle disease virus B1                 A.....GA..T..A..T.....GG.......GGGG..CC.......C..CA........G....G...............   240
Newcastle disease virus strain B1 Takaa    A.....GA..T..A..T.....GG.......GGGG..CC.......C..CA........G....G...............   240
Newcastle disease virus strain Musktesw    A.....GA..T..A.................G.G...C...........CA...................A.........   240
VNDV/CK/EG/VIR-SHABS/2014                  .........................................................................A......   240
Pigeon/Egypt/KFS/2017/2                    .A...........A..A.......A......G..G..C...........CA....C..T.................C..G   240
Pigeon/Egypt/KFS/2017/5                    ......GA..T..T..T.....GG.......GGGG..CC.......C...........GG....G...............   240

Majority                                   ACTGCCGCACAGATAACAGCAGCCGCGGCTCTGATACAAGCCAAACAAAATGCTGCCAACATCCT
                                           ---------+---------+---------+---------+---------+---------+-----
                                                  250       260       270       280       290       300
                                           ---------+---------+---------+---------+---------+---------+-----
NDV-Chicken/China/SDWF07/2011              ACAGCGGCACAGATAACAGCAGCTGCGGCCCTGATACAAGCCAAACAGAATGCCGCCAACATCCT                  305
NDV-H1 Fusion protien                      ..T..C.....A........G..C..A..T.................A.....T...........                  305
NDV-LASOTA-II-fusion                       ..T..C.....A........G..C..A..T.................A.....T...........                  305
NDV- isolate Hitchner fusion genes         ..T..C.....A........G..C..A..T.................A.....T...........                  305
NDV-clone 30                               ..T..C.....A........G..C..A..T.................A.....T...........                  305
NDV--VG/GA                                 ..T..C.....A..GG....G..C..A..T.................A.....T...........                  305
vectormune NDV Strain                      ..C..T.................CT....T..............T..A.....T...........                  305
NDV-chicken/N. Ireland/Ulster/67           ..C..T..................T....T..............C..A.....T...........                  305
NDV-FU13-EGYPT-NLQP-2014                   .....................................................T..........A                  305
pigeon/Egypt/Sharkia-2/2013 fusion         ...T........................................C....................                  305
Newcastle disease virus strain NDV V4      ..C..T.................CT....T..............T..A.....T........A..                  305
Newcastle disease virus VG/GA clone 5      ..T..C.....A........G..C..A..T.................A.....T...........                  305
NDV-avenu                                  ..C..T.................CT....T..............T..A.....T........A..                  305
Newcastle disease virus B1                 ..T..C.....A........G..C..A..T.................A.....T...........                  305
Newcastle disease virus strain B1 Takaa    ..T..C.....A........G..C..A..T.................A.....T...........                  305
Newcastle disease virus strain Musktesw    .....T...........G.....CT....T..............C........T..T........                  305
VNDV/CK/EG/VIR-SHABS/2014                  .....................................................T...........                  305
Pigeon/Egypt/KFS/2017/2                    ...T.A....................A.................T....................                  305
Pigeon/Egypt/KFS/2017/5                    .....C.....A........G..C.....T..T..............A.....TT..........                  305
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Fig (2) Nucleotide sequences alignment of F gene of the isolated PPMV-1 in comparison to some vaccinal and reference strains available in the genbank.

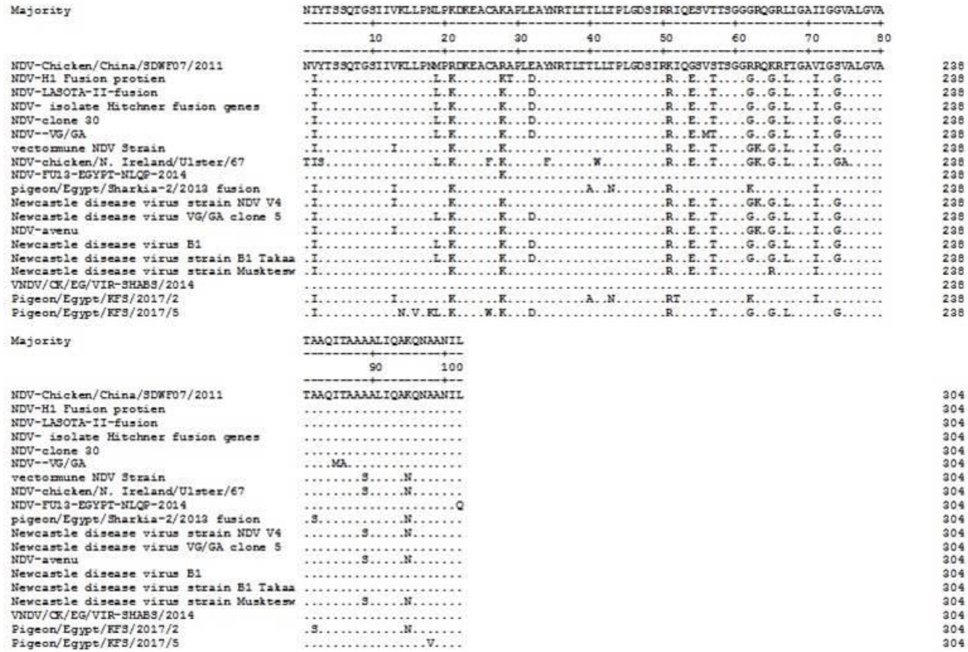

Fig (3) Alignment of deduced amino acid sequences of F protein of the isolated PPMV-1 in comparison to vaccinal and reference strains available in the genbank.

Percent Identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		86.1	87.1	87.1	87.1	84.2	85.1	79.2	99.0	90.1	85.1	87.1	85.1	87.1	87.1	90.1	100.0	88.1	84.2	1	NDV-Chicken/China/SDWF07/2011
2	15.4		99.0	99.0	99.0	96.0	93.1	89.1	87.1	85.1	93.1	99.0	93.1	99.0	99.0	91.1	86.1	85.1	92.1	2	NDV-H1 Fusion protien
3	14.2	1.0		100.0	100.0	97.0	94.1	90.1	88.1	86.1	94.1	100.0	94.1	100.0	100.0	92.1	87.1	86.1	93.1	3	NDV-LASOTA-II-fusion
4	14.2	1.0	0.0		100.0	97.0	94.1	90.1	88.1	86.1	94.1	100.0	94.1	100.0	100.0	92.1	87.1	86.1	93.1	4	NDV- isolate Hitchner fusion genes
5	14.2	1.0	0.0	0.0		97.0	94.1	90.1	88.1	86.1	94.1	100.0	94.1	100.0	100.0	92.1	87.1	86.1	93.1	5	NDV-clone 30
6	17.8	4.1	3.0	3.0	3.0		91.1	87.1	85.1	83.2	91.1	97.0	91.1	97.0	97.0	89.1	84.2	83.2	90.1	6	NDV--VG/GA
7	16.6	7.3	6.2	6.2	6.2	9.5		92.1	86.1	88.1	100.0	94.1	100.0	94.1	94.1	94.1	85.1	88.1	87.1	7	vectormune NDV Strain
8	24.4	11.8	10.6	10.6	10.6	14.2	8.4		80.2	80.2	92.1	90.1	92.1	90.1	90.1	88.1	79.2	80.2	84.2	8	NDV-chicken/N. Ireland/Ulster/67
9	1.0	14.2	13.0	13.0	13.0	16.6	15.4	23.0		89.1	86.1	88.1	86.1	88.1	88.1	91.1	99.0	89.1	85.1	9	NDV-FU13-EGYPT-NLQP-2014
10	10.6	16.6	15.4	15.4	15.4	19.1	13.0	23.0	11.8		88.1	86.1	88.1	86.1	86.1	90.1	90.1	98.0	81.2	10	pigeon/Egypt/Sharkia-2/2013 fusion
11	16.6	7.3	6.2	6.2	6.2	9.5	0.0	8.4	15.4	13.0		94.1	100.0	94.1	94.1	94.1	85.1	88.1	87.1	11	Newcastle disease virus strain NDV V4
12	14.2	1.0	0.0	0.0	0.0	3.0	6.2	10.6	13.0	15.4	6.2		94.1	100.0	100.0	92.1	87.1	86.1	93.1	12	Newcastle disease virus VG/GA clone 5
13	16.6	7.3	6.2	6.2	6.2	9.5	0.0	8.4	15.4	13.0	0.0	6.2		94.1	94.1	94.1	85.1	88.1	87.1	13	NDV-avenu
14	14.2	1.0	0.0	0.0	0.0	3.0	6.2	10.6	13.0	15.4	6.2	0.0	6.2		100.0	92.1	87.1	86.1	93.1	14	Newcastle disease virus B1
15	14.2	1.0	0.0	0.0	0.0	3.0	6.2	10.6	13.0	15.4	6.2	0.0	6.2	0.0		92.1	87.1	86.1	93.1	15	Newcastle disease virus strain B1 Takaa
16	10.6	9.5	8.4	8.4	8.4	11.8	6.2	13.0	9.5	10.6	6.2	8.4	6.2	8.4	8.4		90.1	90.1	85.1	16	Newcastle disease virus strain Musktesw
17	0.0	15.4	14.2	14.2	14.2	17.8	16.6	24.4	1.0	10.6	16.6	14.2	16.6	14.2	14.2	10.6		88.1	84.2	17	VNDV/CK/EG/VIR-SHABS/2014
18	13.0	16.6	15.4	15.4	15.4	19.1	13.0	23.0	11.8	2.0	13.0	15.4	13.0	15.4	15.4	10.6	13.0		81.2	18	Peagon/Egypt/KFS/2017/2
19	17.8	8.4	7.3	7.3	7.3	10.6	14.2	17.8	16.6	21.7	14.2	7.3	14.2	7.3	7.3	16.6	17.8	21.7		19	Pigeon/Egypt/KFS/2017/5
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Fig (4) Amino acids identity and divergence of the isolated PPMV-1 in comparison to vaccinal and reference strains available in the genbank.

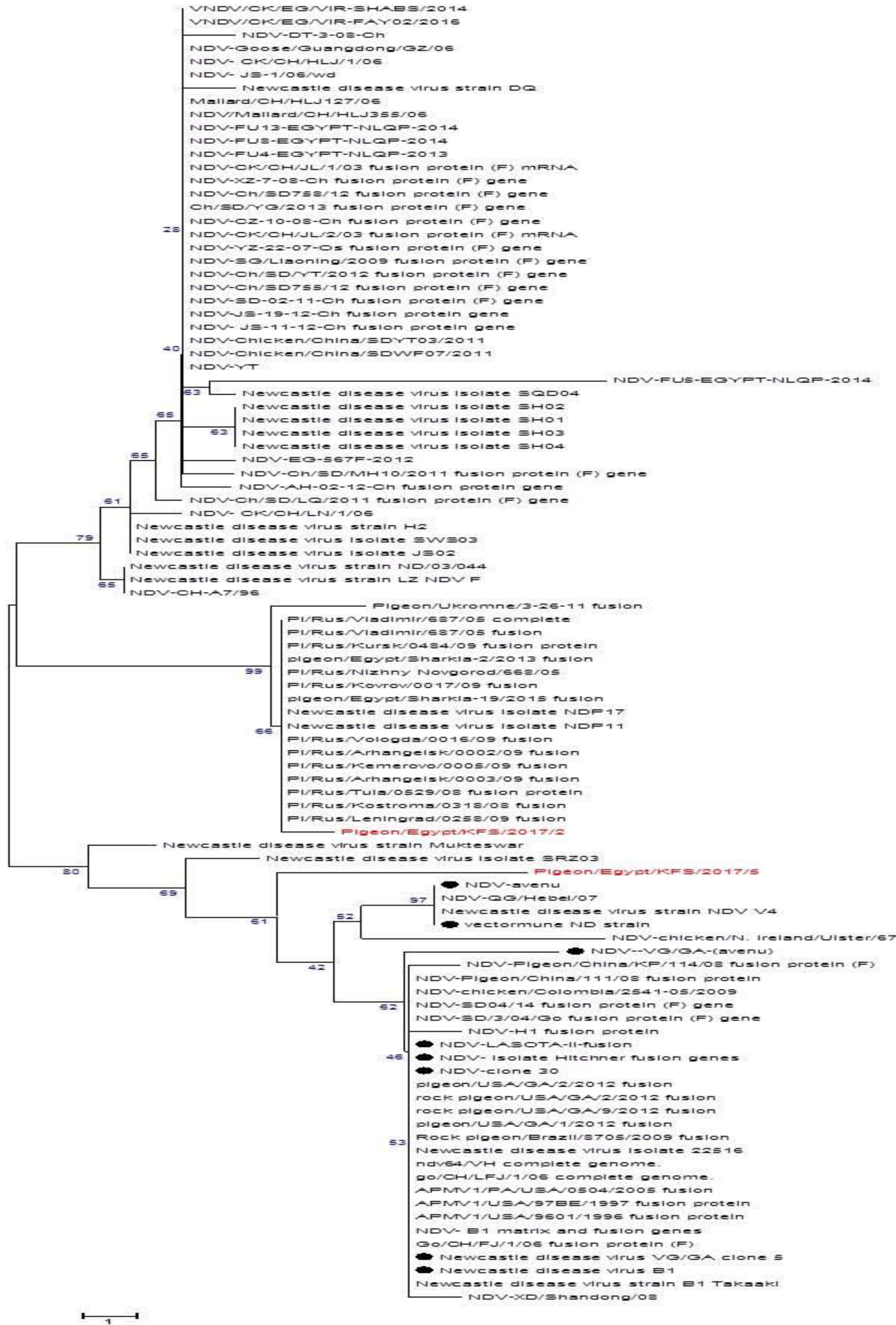

Fig (5) Phylogenetic tree based on F protein partial amino acid sequence of selected isolates and published sequences. (*) Marked strains refer to commercially available vaccinal strains.

. When HI test was performed using Lasota and Hitchner vaccinal strains as an antigen, HI titers were 2^9 and 2^{12} respectively after proper titration of viral antigen. The higher titer obtained when Hitchner strain was used as an antigen indicates that it is more compatible to the strain from which antiserum had been prepared and therefore is more convenient for vaccination when compared to Lasota strain. The same result was reported by (Eskelund, 1986). However other authors as (Guo *et al*., 2013) reported that the commercially usable ND vaccines as Lasota and Hitchner may not provide effective protection for APMV-1 infection in pigeons.

HI positive samples were subjected to RT-PCR which approved the results of virus isolation and identification (Pestka *et al*., 2014), where all 12 positive samples show specific size bands (356 bp) on the agar gel.

The molecular characterization and phylogenetic analysis are of great importance to detect viral evolution and disease outbreaks and to enhance the control measures (Ewies *et al*., 2017). Alignment of nucleotide and deduced amino acid sequences of F gene of the isolated PPMV-1 in comparison to some vaccinal and reference strains available in the genbank revealed that the 1st APMV isolate in this study (Pigeon/Egypt/KFS/2017/2) showed identity percent ranged between 80.2–98% (Fig 4 and 5) while the 2nd isolate (Pigeon/Egypt/KFS/2017/5) showed identity percent ranged between 81.2-93.1%. Compared to previous Egyptian isolates, nucleotide and deduced amino acid sequence analysis revealed a low rate of mutation between isolate (Pigeon/Egypt/KFS/2017/2) and previous published Egyptian isolate (Pigeon/Egypt/Sharkia-2/2013), with only two amino acids substitution at positions K78R and T102I.

On the other side, 2nd isolate in this study (Pigeon/Egypt/KFS/2017/5) was distinct from previous isolates showing 81.2% identity with (Pigeon/Egypt/KFS/2017/2) and (Pigeon/Egypt/Sharkia-2/2013). However, it showed higher identity (93.1%) with the ND vaccinal strains as Hitchner, Lasota and Clone 30.

The F protein phylogeny revealed that the isolate (Pigeon/Egypt/KFS/2017/2) was classified as genotype VIb (class II) which is the classical genotype of APMV-1 (Guo *et al.,* 2014) and within sublineage VIb/2. It was closely related to the Egyptian isolates such as (Pigeon/Egypt/Sharkia-2/2013) (accession no. KX580976) and (Pigeon/Egypt/Sharkia-19/2015) (accession no. KX580988) as they were clustered together in the same genotype (Fig 5). However, the isolate (Pigeon/Egypt/KFS/2017/5) was distinct from other isolates and formed a single clade closer to the vaccinal strains. This isolate may originated from repeated mutations or natural homologous recombination which resulted in amino acid motif at the F0 cleavage site closer to lentogenic strains (^{112}GRQGRL^{117}). This suggestion is confirmed by (Wang *et al.,* 2015) where they reported that live vaccinal strains may play roles in shaping NDV evolution by homologous recombination with circulating viruses where the original parents were derived from vaccine strains and circulating virus strains.

Based on the phylogenetic analyses, the genotype VIb isolates of NDV from pigeons showed low amino acid identity with vaccinal strains Hitchner and Lasota (genotype II) and V4 (genotype I), and in this study, we found that the HI titre using Lasota and Hitchner as antigens was higher than when using APMV-1 isolates where the variations in HI antibody titers rely on the virus strain used as an antigen in the HI assay (Dortmans *et al*., 2011). This indicates that these amino acid substitutions in neutralizing epitopes have converted the antigenicity of PPMV-1 (Mase and Kanehira, 2015). Thus the current usable vaccines may not succeed to offer sufficient immunity for pigeons against genotype VIb viral strains. It is strongly recommended that vaccines for pigeons should be prepared from PPMV-1 virus which had been isolated from of pigeons and based on VIb (Alexander *et al.,* 1986).

Infectivity of NDV is based on the post-translational cleavage of non-infectious F protein precursor (F0) into F1 and F2. Virulent strains of NDV have multiple basic amino acids (3 arginine-R and lysine-K between residues 113-116 and phenylalanine at residue 117) at the proteolytic cleavage site of F0 precursor. This cleavage site is susceptible to cleavage by the host ubiquitous protease found all over the body (Nidzworski *et al.,* 2011). In addition, the phenylalanine (F) at position 117 has shown to be a possible contributor to the nervous effects (Collins *et al*., 1993). However other authors suggest that F protein is not the only determinant of virulence and they suggest the contribution of other genes, like HN protein gene (Huang *et al*., 2004; Leeuw *et al*., 2005).

Pathotyping of APMV-1 isolates with conventional tools such as ICPI, IVPI and is difficult and laborious, but new methods such as nucleotide

sequence analysis of F gene encoding the cleavage site are rapid and sensitive tools. (Mayahi et al., 2017)

In this study, molecular pathotyping was performed based on the amino acid sequences of the cleavage site residues (112 to 117) of the F0 protein and the results confirmed that the 1st isolate (Pigeon/Egypt/KFS/2017/2) possesses the F0 cleavage site motif ^{112} KRQKRF^{117} which is characteristic for the velogenic strains .Previous studies have shown that the sequence of the cleavage sites of PPMV-1 which evolved: ^{112}GRQKRF^{117} (was present in PPMV-1 isolates from the 1980s), has been replaced by ^{112}RRQKRF^{117} from the beginning of the 1990s which seems to be predominant in recent years in most countries (Śmietanka K, Minta, 2011). However this motif (^{112} KRQKRF^{117}) has also been reported in other Egyptian isolates (Mansour *et al.,* 2017). Our 2nd isolate (Pigeon/Egypt/KFS/2017/5) possesses amino acids motif (^{112}GRQGRL^{117}) which is characteristic for lentogenic strains which is in agreement with (OIE, 2012) which reported that the detection of NDV without multiple basic amino acids at the (F0) proteolytic cleavage site by RT-PCR does not certify the absence of a virulent virus whereas the multiple basic amino acids ensures the existence of a virulent virus. (Wang et al., 2015) and (Qiu et al., 2017) isolated PPMV-1 strains with a virulence motif of 112RRQKRF117 in the F protein from symptomless pigeons suggesting that PPMV-1 isolates tended to develop a symbiotic relationship with pigeons so that infected pigeons have no clinical signs but still being carriers and shed the virus occasionally.

5. CONCLUSIONS

In conclusion, there are different APMV-1 circulating in the Egyptian fields which are not closely related to the commercially available vaccines and therefore it is strongly recommended to develop and apply advanced diagnostic and control measures including effective vaccination programs using vaccines based on VIb viruses in Egypt to safeguard pigeon's health to reduce the economic losses.

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