Research Article

Genetic and Pathogenic Characterization of a Newcastle Disease Virus Isolated from Pigeons in Egypt

Ahmed Samy[1]*, Hala Mohamed Nabil Tolba[2], Gamelat Kotb Farag[3] and Ahmed Abd El Halim[1]

[1]Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza 12618, Egyp; [2]Department of avian and rabbit diseases; [3]Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Abstract | Genotype VI Newcastle disease viruses (NDV), that designated pigeon paramyxovirus 1 (PPMV1) commonly isolated from pigeons (family Columbidae). PPMV1 not only represent a potential threat to pigeon but also it can infect chickens and its virulence enhanced upon passaging in chickens causing clinical signs and deaths. In the present study brain samples were collected from twelve pigeon flocks exhibiting signs of nervousness and diarrhea in a live bird market (LVM) in Egypt. All collected samples were positive for NDV and negative for avian influenza virus and infectious bronchitis virus. Seven positive samples were subjected for genotypic characterization based upon partial F gene sequencing. Partial F gene sequencing revealed that all tested isolates harbor a cleavage site with multi basic amino acid ^{112}KRQKR/F^{117} characteristic of velogenic strains. However, phylogenetic analysis revealed clustering of all tested strains within sub-genotype VIg. Three isolates were assessed for pathogenicity based upon the mean death time (MDT), the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) in chicken. The ICPI and MDT revealed that all tested isolates were of moderate virulence (Mesogenic) in chickens. Mature chickens showed no clinical signs or death as assessed by IVPI. Pigeon in Egypt reared in free rang system and sold in LBM mostly for restocking, that with extensive infection with PPMV represent a potential threat to chickens. Therefore, strict biosecurity measures and control measures at live bird markets, alongside the development of a vaccine may be required to reduce the risk of PPMV1 outbreaks.

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***Correspondence** | Ahmed Samy, Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza 12618, Egyp; **Email:** dr.ahmed189@gmail.com
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Introduction

Newcastle disease severely affects the poultry industry worldwide, with severe losses even in the presence of an intensive vaccination programme (Jeon Lee et al., 2008; Rui Juan et al., 2010; Diel Susta et al., 2012). Newcastle disease is caused by Newcastle disease virus (NDV), a member of the genus Avulavirus within the family Paramyxoviridae and is designated avian paramyxovirus-1 (APMV-1) (Mayo, 2002). NDV is an enveloped, non-segmented, negative sense, single stranded RNA virus of approximately 15,186 nucleotides that encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin neuraminidase (HN), and the RNA polymerase (L) (Lamb, 1996). The M, F and HN proteins associated with the viral envelope mediate the entry and release of the NDV. However,

the amino acid sequences of the F protein cleavage site represent the key molecular determinant of NDV virulence (Glickman Syddall et al., 1988). NDV strains can be classified into three categories: velogenic, mesogenic, and lentogenic strains that induce acute, moderate and mild symptoms respectively based upon standard biological tests as recommended by the OIE, such as the mean death time (MDT) in embryonated chicken eggs, the intracerebral pathogenicity index (ICPI) in 1-day-old chicks, and the intravenous pathogenicity index (IVPI) in 6- week-old chickens. (OIE, 2012). Based upon phylogenetic analyses of the nucleotide sequences of the F gene, NDV has been categorized in to two classes; class I contains a single genotype whereas class II contains 15 genotypes (I – XV) with sub-genotypes in both cases (Diel da Silva et al., 2012). Columbiformes (pigeons and doves) are primarily infected by genotype VI-PMV1, also known as pigeon paramyxovirus 1 (PPMV-1), which is antigenically and genetically distinguishable from Avian PMV-1 (APMV-1).

PPMV-1 emerged for the first time in the middle east in the late 1970s (Lister Alexander et al., 1986). Subsequent it the initial emergence, there has been a continuing panzootic recorded frequently worldwide (Aldous Fuller et al., 2014), primarily in pigeons and doves, but additionally other wild birds and poultry (Alexander Wilson et al., 1984). Typically, PPMV-1 isolates mesogenic in chickens. However, passaging of the virus in in chickens has resulted in an increase in virulence and replication based upon ICPI values. Furthermore, only three mutations in two viral genes, P and L, are necessary to result in increased virulence and enhanced replication in chickens (Dortmans Rottier et al., 2011). Therefore PPMV-1 represents a potential threat to poultry production (Aldous Fuller et al., 2004; Krapež Račnik et al., 2010). In pigeons and doves, infection with PPMV-1 causes systemic infection resulting in respiratory manifestations (Toro Hoerr et al., 2005), central nervous system disorders (tremor of the neck and wings, torticollis, paralysis and disturbed equilibrium) and symptoms associated with gastrointestinal tract disorders (excessive drinking and watery to hemorrhagic diarrhea) (Alexander and Parsons, 1986).

In Egypt, pigeons are mainly raised for meat consumption and kept in pigeon towers with direct contact to household birds and near commercial farms. The pigeon trade in Egypt is centers largely on live bird markets, where other domestic birds from the household and commercial sectors are also present. In the present study, samples were collected from live pigeon markets in Egypt from pigeons with nervous manifestations and with whitish green diarrhea.

Materials and Methods

Ethics statement

The experimental design and protocols used in the present study was reviewed and approved by laboratory biosafety and ethics committee of reference laboratory for quality control on poultry production (RLQP) to ensure the safety of the environment and minimizing the pain and discomfort to the animal while conducting the experiments. All virus isolation and animal experiments were performed in virology unit and biological experiments and research unit at RLQP under strict control biosafety level 3 facilities.

Sample collection, processing and confirmation of NDV presence

Brain samples were collected from 12 pigeon farms harbouring birds showing with severe neurological symptoms (Table 1). The brain was removed under aseptic conditions and placed in viral transport medium (VTM) and transferred to the laboratory for further investigation. Brains samples were homogenized with in equal volume of phosphate buffer saline. The homogenate was vortexed and centrifuged at 1260xg for 30 min. after which the supernatant was collected for further processing.

Total RNA was extracted from the brain homogenate using the QIAamp Viral RNA Mini Kit (QIAGEN) in accordance with manufacturer's instructions. Genetic material was amplified using Quanti Tect probe RT-PCR kit (Qiagen), primers and thermal profile as previously described by (Wise Suarez et al., 2004) to detect NDV. Furthermore, the extracted RNA was also screened using real time RT-PCR for presence of avian influenza (AI) H5, H7, H9, and avian corona virus using primers and thermal profiles as previously described (Slomka Pavlidis et al., 2007); (Shabat Meir et al., 2010), (Callison Jackwood et al., 2001).

Virus isolation propagation and pathotyping

The brain homogenates of all samples positive for the presence of NDV by real time PCR were filtered using 0.45 µm pore size (Millex® - Sigma Aldrich) 0.2ml of filtered brain homogenate was inoculated

Table 1: *Background data for the collected samples.*

0	Locality	Breed	Age	Total No	Vaccine	Mortality	Clinical signs	P.M[2] lesions	ICPI	Cleavage site	Accession No.
1	Zagazig	Baladi	1.5	300	Lasota	20	Twisting of neck, unable to fly, Greenish diarrhea	Septicemia with congested brain	1.41	KRQRF	MH723605
2	El-Shobak	Baladi	9m	50	Lasota	10			ND	KRQRF	MH723606
3	Zagazig	Romani	7m	13	---[1]	5			ND	KRQRF	MH723607
4	Abuhamad	baladi	4m	35	---	4		Septicemia with congested brain+ Ascaris	ND	KRQRF	MH723608
5	Zagazig	Remaiah	2y	100	Lasota	30		Septicemia with congested brain	ND	KRQRF	MH723609
6	Zagazig	Remaih	2.5y	140	---	10			ND	ND	ND
7	Hehia	Baladi	7m	50	---	6	Move in circle, conjunctivitis, Anorexia ,greenish diarrhea	Septicemia with congested brain	ND	ND	ND
8	Miny-aEL-Qamh	Australy	9m	160	Lasota	20	Torticollis, twisting of neck, greenish diarrhea		1.51	KRQRF	MH723610
9	Zagazig	Baladi	2y	100	---	15			ND	-----	
10	Zagazig	Baladi	4m	30	---	5			1.48	KRQRF	MH723611
11	EL-mesal-mia	Baladi	6m	60	---	2		Septicemia with congested brain+cestode infestation	ND	ND	ND
12	Hehia	Baladi	3m	20	---	2	Off food, lameness, unable to fly, greenish watery diarrhea	Congested brain with slight congestion in parenchymatous organ	ND	ND	ND

1: *no available data;* **2:** *post mortem;* **3:** *not detected.*

into 9-day old specified pathogen free (SPF) embryonated chicken eggs (SPF farm, Koam Osheim, El-Fayoum, Egypt) following standard procedures (Alexander et al., 1998). SPF eggs that remained alive after the first 24 hours were candled daily for 5 days for signs of death. The dead embryos were chilled overnight at 4˚C to collect allantoic fluid. The allantoic fluid was tested for haemagglutination activity by heamagglutination (HA) and heamagglutination Inhibition (HI) tests using four HA units according to reference procedure (OIE, 2012). The egg infective dose 50 (EID50) was calculated according to (Reed and Muench, 1938). The virulence of three selected isolates (Egy. PPMV1,8 and10) was evaluated using MDT in 9-day old embryonated chicken eggs and ICPI in one day old SPF chicks in accordance with reference procedures (OIE, 2012).

Sequence and phylogenetic analysis of the fusion protein gene

Viral RNA was extracted directly from the Allantioc fluid of seven isolates using QIAamp viral RNA mini kit (QIAGEN, Germany) in accordance with the manufacturer's instructions. A previously described forward primer (NDV-M2) 5_-TGG AGC CAA ACC CGC ACC TGCGG– 3(980 to 1003 of the M gene) (Mase Imai et al., 2002), and a reverse primer (NDV-R960) 5_CGG GAC TAG TGC TGA GGC ATA TCC TTT GG-3 that has been developed in RLQP were synthesized by Bio Basic (Ontario, Canada). RT-PCR was performed using Sensi FAST™ One-Step Real-Time PCR (Bio line). The cycling conditions were: 45◦C for 20 min at RT, 95 ◦C for 2 min and 30 cycles of 95◦Cfor 30 Sec., 52◦C for 45Sec., and 72 ◦C for 45 Sec., followed by 72 ◦C for 10min. PCR products were purified using the QIA quick gel extraction kit (Qiagen, USA), according to the manufacturer's instructions. The purified PCR products were sequenced by Applied Bio systems 3130 genetic analyzer (Hitachi, Japan) using Big dye Terminator V3.1 cycle sequencing chemistry (Perkin-Elmer, Foster city, CA, USA) according to the manufacturer's instructions. BioEdit v7.0 (Hall, 1999) was used for the alignment of nucleotide and predicted amino acid sequences. Sequences representing different NDV genotypes and sub genotype VI were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) and aligned with strains of the present study. Maximum likelihood phylogenetic trees were constructed using MEGA version 5 with 1000 bootstrap replicates. (Tamura Peterson et al., 2011). Trees were viewed and edited using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software /figtree/).

Sequences of the partial F gene of the seven isolates in the present study have been submitted to GenBank with the accession numbers provided in Table1.

Results and Discussion

Clinical findings and PCR confirmation

Clinical signs, gross lesions, mortality, morbidity and vaccination status of the collected birds are summarized in Table 1. Nervous signs and diarrhea were observed in the majority of cases. Septicemia and congested brain were the most frequently observed gross lesions. No correlation between vaccination status and incidence of the disease has been observed. Based upon real time RT-PCR results, all collected samples were positive for virulent NDV and negative for AIV-H5, H7, H9 and Avian corona virus.

Virus isolation and pathogenicity

NDV was successfully isolated from all twelve collected samples and confirmed as NDV by HA, HI and real time RT-PCR. Three isolates were selected for further analyses of pathogenicity. The ICPI values of the selected isolates (PPMV1, 8 and10) were 1.41, 1.48 and 1.44 respectively and the MDT was 69, 64 and 67 hours respectively. No overt clinical signs were recorded in any of the 10 chickens for each virus throughout 10 days of observation, indicating zero IVPI. These indices revealed that the tested ND viruses were of moderate virulence (Mesogenic) in chickens (Swayne, 1998).

Sequence and phylogenetic analyses

Partial F gene nucleotide sequences of the seven selected viruses were deposited into the Genbank database with the accession numbers listed in Table 1. All sequenced viruses bear a multi basic amino acid (KRQKR) cleavage site at position 112 to 116 and a phenylalanine residue at position 117 (Table 1). This motif is one of the characteristic motifs of velogenic isolates (Liu Wang et al., 2006).

The phylogenetic analysis of the seven viruses in the present study, along with ninety-seven strains retrieved form GenBank represent different NDV genotypes, revealed that all seven viruses clustered with NDV genotype VI (Figure 1). Furthermore, Phylogenetic analysis of the seven viruses, along with sixty nine strains representing different genotype VI NDVs revealed that all tested viruses clustered within the genotype VIg clade (Figure 2).

In Egypt, APMV represents a major threat to poultry production, despite extensive vaccination strategies and control measures used (Saad Samy et al., 2017). Pigeons represent a potential source for NDV infection for the household and commercial poultry sectors (Rohaim El Naggar et al., 2016). Traditional live bird markets (LBM) in lack food safety standards and an absence of veterinary inspection (Abdelwhab Selim et al., 2010). Further, LBMs represent the main interface of different bird's species allowing the spread of infection, particularly as birds are traded at LBMs for restocking rather than consumption (FAO, 2009). In a previous study, samples from pigeons with nervous manifestations and diarrhea were collected from El Sharkia governorate during 2013 to 2015 and tested for the presence of avian influenza and NDV, in turn confirming the co-circulation of paramyxo- and influenza viruses in pigeons (Mansour Mohamed et al., 2017).

In the current study, samples representing twelve flocks were collected and confirmed to be NDV positive, whilst being negative for AIV and IBV with mortality rate ranged from 3.3 to 38.5%. The mortality rate (3.3-38.5% among all listed flocks with nervous signs and greenish diarrhea) was low relative to the previous study describing NDV in pigeons (Mansour Mohamed et al., 2017), where the mortality rate ranged from 10 to 92.5%. This difference may be attributable to the absence of the mixed infection with AIV.

Based on IVPI and MDT results, all of the tested isolates in the present study were considered as moderately virulent (Mesogenic) to chickens, with an inability to induce any clinical signs when injected intravenously into six-week-old SPF chickens, these finding go in context with previous studies (Sabra Dimitrov et al., 2017) and (Liu Qu et al., 2015). Despite the fact that the PPMV1 strains assessed in the present study are considered as mesogenic, they are still considered to be a potential source of NDV infection for commercial and household poultry, particularly considering that the virulence of PPMV1 was enhanced upon serial passage in chickens (Kommers King et al., 2003; Dortmans Rottier et al., 2011). This serial passage is widely expected due to the direct contact between pigeons that are reared free range in Egypt and sold live at LBMs in the presence of other poultry species.

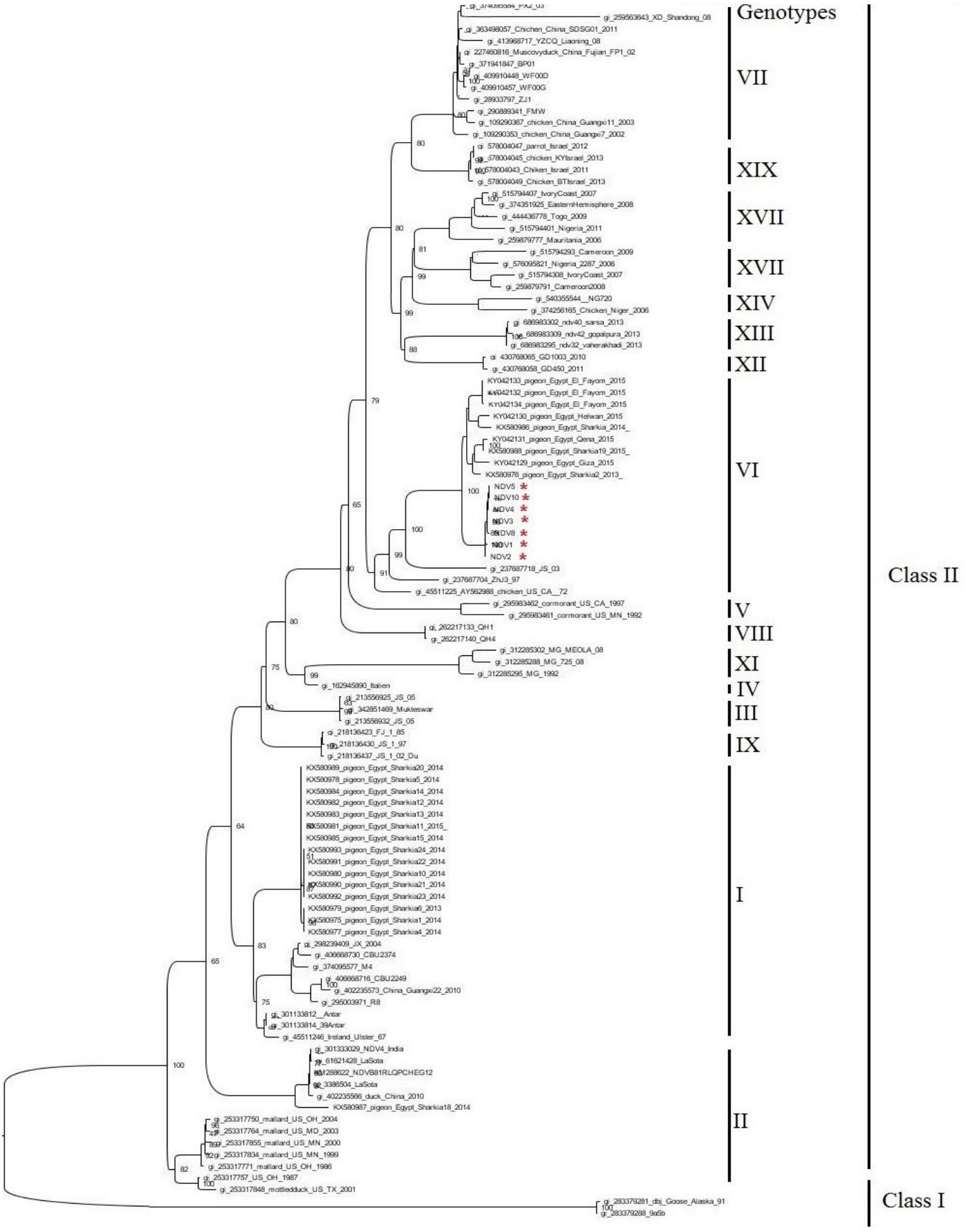

Figure 1: *Phylogenetic analysis based on partial F gene sequencing. Isolates used in the present study highlighted with star along with Sequences represent different NDV genotypes. The phylogenetic tree was constructed by a maximum likelihood method with 1000 bootstrap replicates (bootstrap values are shown on the tree).*

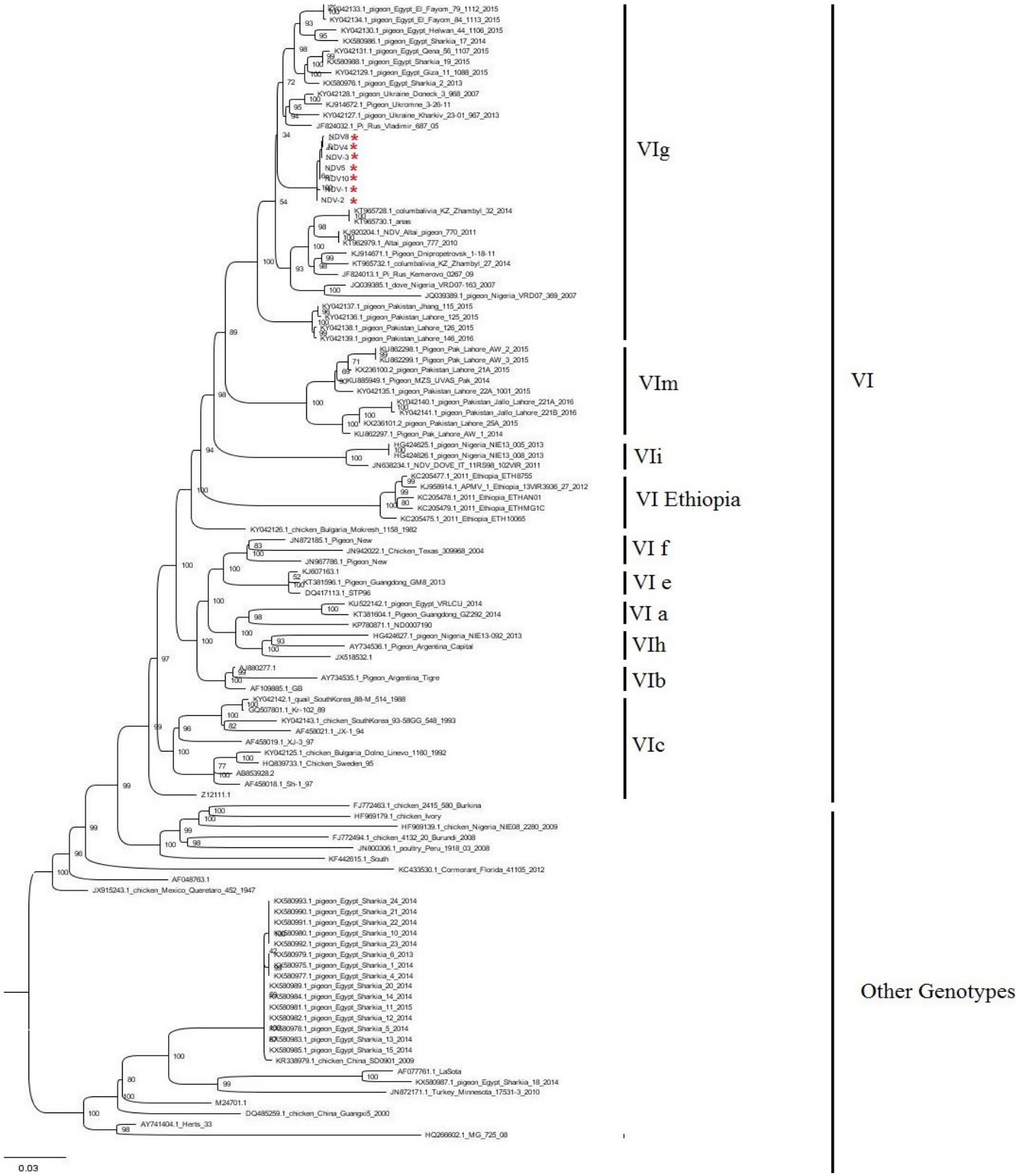

Figure 2: *Phylogenetic analysis of the partial F gene sequencing. Isolates used in the present study are highlighted with a star. Along with Sequences represent different NDV VI sub genotypes. The phylogenetic tree was constructed by a maximum likelihood method with 1000 bootstrap replicates (bootstrap values are shown on the tree).*

The F protein of the seven isolates examined in the present study contained the same polybasic cleavage site motif (KRQKR) that is typical of virulent strains and appears to be the dominant cleavage site motif for PPMV1 viruses circulated among pigeons in Egypt (Sabra Dimitrov et al., 2017). It is worth note that gradual passage of avirulent NDV strain with cleavage site motif (ERQER) in chickens, the avirulant NDV strain become more efficiently replicating in all organs including brain and cleavage site

motif changed to be (KRQKR) (Shengqing Kishida et al., 2002). Furthermore, isolates virulent in pigeons and quail with polybasic cleavage motif become highly virulent upon passaging in chickens, (Alexander and Parsons, 1986; Islam ITO et al., 1995).

Phylogenetic analysis of the isolates sequenced in the present study revealed that they all clustered within genotype VI NDV sub genotype VIg. Isolates of the same genotype have previously been recovered from oropharyngeal and cloacal swabs collected from healthy pigeons in Egypt (Sabra Dimitrov et al., 2017).

In conclusion, we isolated PPMV1 from the brain of pigeons exhibiting clinical signs in a live bird market in Egypt. Although the viruses are of moderate virulence as assessed using IVPI and MDT they initially caused no clinical signs in chickens, the virulence gradually increased upon passage in chickens. As a result, the potential of PPMV1 to induce losses among chickens must not be underestimated and the virus must be controlled within the pigeon population via improved vaccination and biosecurity measures.

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Author's Contributions

Hala M.N. Tolba and Gamelat. K. Farag collect and prepare the clinical samples and data from life bird markets. Ahmed Samy designed the study, performed the methodology, evaluated the results and wrote the manuscript. Ahmed Abd El Halim performed PCR confirmation. All author read and approved the final version of the manuscript for submission.

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