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Evidence for Possible Transmission of Newcastle Disease Virus of Sub-Genotype XXI.1.2 Between Feral Eurasian Collared Doves and Backyard Chickens in Balochistan Province, Pakistan

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Key words
Backyard chickens, Feral Eurasian collared doves, Genome sequencing, PPMV-1, Subgenotype XXI.1.2

ABSTRACT

In October 2020, two feral Eurasian collared doves (*Streptopelia decaocto*) were found dead near a backyard chicken flock. Brain and trachea tissues were collected from the dead collared doves and swab samples were collected from 42 healthy in-contact backyard chickens for the isolation of Newcastle disease virus (NDV). Full-length genomic sequences and phylogenetic analysis of four viruses (dove = 2, chicken = 2) showed that they belonged to sub-genotype XXI.1.2 and were highly similar (99.92%) to each other identifying possible spillover of viruses between domesticated and wild bird populations. The findings provide further insights into the potential influence of feral collared doves on NDV epidemiology.

INTRODUCTION

Newcastle disease (ND) is one of the most infectious viral diseases infecting a wide range of poultry and non-poultry avian species and has an immense economic impact on poultry trade and food security globally (Wajid *et al*., 2017). ND is endemic in Pakistan poultry production facilities (Irshad *et al*., 2020). Newcastle disease virus (NDV) also known as Avian orthoavulavirus 1 (AOAV-1) belongs to the genus *Orthoavulavirus* in the family Paramyxoviridae. The viruses are classified into two classes (I and II) consisting of a single and 21 genotypes, respectively, based on full-length fusion (*F*) gene sequences (Dimitrov *et al*., 2020). The viral genome has single-stranded and negative-sense RNA of approximately 15,200 base pairs (bp) and contains six transcriptional units in the order of 5-NP-P-M-F-HN-L-3 (Ather *et al*., 2023). Pigeon paramyxovirus 1 (PPMV-1) is an antigenic variant of NDV that is commonly isolated from wild and domestic pigeons and doves and occasionally spillovers into poultry (Wajid *et al*., 2016). Genotypes XX, XXI, and VI of mesogenic and velogenic PPMV-1 pathotypes are the most frequently isolated from wild and domestic Columbiformes worldwide (Nasir *et al*., 2022). Although the viruses in these genotypes cause high morbidity and mortality in pigeons and doves, they only cause mild to moderate respiratory and neurological signs in non-Columbidae species (Abolnik *et al*., 2008; Aziz-ul-Rahman and Shabbir, 2019; Krapez *et al*., 2010; Nooruzzaman *et al*., 2021). PPMV-1-ND outbreaks in chickens have been reported in several European and African countries (Dodovski *et al*., 2013). Previous studies have reported that the persistence of PPMV-1 strains in poultry production facilities can result in the development of strains virulent to poultry (Hussain *et al*., 2020). Our recent study identified the maintenance of PPMV-1 strains (genotype XXI.1.2) in a live bird market

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(LBM) in the Lahore district (Dodovski *et al*., 2013) and similar viruses have been isolated from wild/domestic pigeons and commercial poultry farms (Hussain *et al*., 2020; Wajid *et al*., 2017). In this current study, we have sequenced the complete genome of two PPMV-1 isolated from feral Eurasian collared doves in Pakistan to establish their relationship to viruses isolated from healthy backyard chickens.

MATERIALS AND METHODS

In October 2020, two feral Eurasian collared doves were found dead in an area shared with backyard chickens in the Pishin district, Balochistan province, Pakistan. Brain and trachea tissues of the dead doves were collected and frozen at -80°C until used for virus isolation as per previously established protocols (OIE, 2012). The dead doves were clinically examined and post-mortem observations were recorded. Post-mortem examination of the collared doves revealed ND-like gross lesions including hemorrhages in the proventriculus, trachea and lungs, and brain hyperaemia. Forty-two (42) healthy backyard chickens (*Gallus gallus domesticus*) in a flock that shared feed and water with the wild doves were sampled for the isolation of PPMV-1. Oropharyngeal swabs were collected in cryovials with 500 µL viral transport media (5%-glycerol-MEM media pH 7.0 supplemented with 200mg streptomycin/mL, 2000IU penicillin/mL, 250 mg gentamicin/mL, 2.5 mg amphotericin B per mL) as per recommended protocol (OIE, 2012) and kept on ice for transport to the laboratory.

The tissues collected from the dead doves were homogenized in 500 µL phosphate buffer saline (PBS) along with oropharyngeal swabs collected from healthy laying hens were centrifuged at 4500 rpm for 20 min, and the supernatants were filtered through 0.22µm sterile filters. The filtrate (100 µL) was inoculated into 11-day-old embryonated chicken eggs (ECEs, free from specific NDV antibodies) and incubated at 37°C for a maximum of five days with daily monitoring. After post-inoculation (pi), the harvested allantoic fluids were subjected to rapid hemagglutination assay (HA) by using 1% chicken red blood cells (RBCs). The positive allantoic fluids were frozen at -80°C until further use.

Briefly, the HA-positive infected allantoic fluids of the brain, trachea, and swabs samples were processed for total RNA isolation by using TRIzol LS reagents (Invitrogen, USA) following the manufacturer's recommendations and subjected to cDNA synthesis employing random hexamer primers according to the recommended protocol. The full-length genome sequencing of the selected four isolates (from dove: n = 2 and backyard chicken n = 2) was performed using overlapping primers as described previously (Wajid *et al*., 2016). This research work on animals was approved by the Departmental Ethical Research Committee of the Virtual University of Pakistan, with approval number 005-16.

To understand the genetic relationship between the PPMV-1 isolates (WECD/121/2020, WECD/122/2020, BC/139/2020, and BC/47/2020) and previously characterized viruses from other countries including Pakistan, a phylogenetic tree was constructed using the maximum likelihood method based on the Tamura-3 model with 1000 bootstrap replicates in MEGA v6 (Tamura *et al*., 2013). The analysis was performed based on full-length fusion (F) genes (n = 71) and genome sequences (n = 47) that included genotypes XXI, XX, and VI associated with pigeons. The obtained full-length genome sequences were submitted to GenBank and are available under the accession number OQ295860 to OQ295863.

RESULTS AND DISCUSSION

The full-length genome of the studied isolates was 15,192 nucleotides (nt), following the rule of six, and had the order 5′-NP (1752)-P (1451)-M (1241)-F (1792)-HN (2002)-L (6703)-3′ with 5′ leader (55 nt) and 3′ trailer (114 nt) sequences. The F-gene cleavage site motif was ^{112}-RRKKRF-^{117} at the C terminus of the F2 protein, a characteristic of virulent NDV strains (Table I). The preliminary BLAST analysis using the F-gene sequences showed that all the isolates had the highest identity with sub-genotype XXI.1.2. The constructed phylogenetic trees confirmed that WECD/121/2020, WECD/122/2020, BC/139/2020, and BC/47/2020 strains clustered into sub-genotype XXI.1.2 and exhibited close genetic relationship (99.95% to 99.96%) with previously characterized viruses isolated in Pakistan between 2014 and 2018 (Fig. 1). The nucleotide distance showed that the viruses isolated from collared doves and backyard chickens were genetically very similar (Table II). A second phylogenetic tree was constructed based on complete genome sequences of the studied isolates with other pigeon associated NDV isolated from various Asian, Middle-East, African and European countries having a similar topology to that based on the complete F gene sequences (Fig. 2).

In this study, the full-length genome of four PPMV-1 isolates was sequenced to explore possible spillover of viruses from feral collared doves to in-contact free-range backyard chickens. The backyard chickens located close to the dead collared doves did not exhibit clinical signs despite the presence of highly similar sub-genotype XXI.1.2 viruses in oral and fecal swab samples. The presence of virus in the swab samples of the chickens

Table I. Description of samples collected and analyzed in this study.

Isolation	Year	Location	Host	Scientific name	Health status	Age	Cleavage site	Geno-type	Genome length (nt)	GenBank
WECD-121	2020	Pishin, Balochistan	Dove	*Streptopelia decaocto*	Dead	-	^{112}-RRKKRF-^{117}	XXI.1.2	15,192	OQ295860
WECD-122	2020	Pishin, Balochistan	Dove	*Streptopelia decaocto*	Dead	-	^{112}-RRKKRF-^{117}	XXI.1.2	15,192	OQ295861
BC-139	2020	Pishin, Balochistan	Backyard chicken	*Gallus gallus domesticus*	Healthy	13M	^{112}-RRKKRF-^{117}	XXI.1.2	15,192	OQ295862
BC-47	2020	Pishin, Balochistan	Backyard chicken	*Gallus gallus domesticus*	Healthy	9M	^{112}-RRKKRF-^{117}	XXI.1.2	15,192	OQ295863

Table II. Estimate of evolutionary distances between the studied strains isolated in this study and different sub-genotypes associated with pigeons.

Studies strains/ genotypes	No. of base substitution per site									
	WECD-121	WECD-122	BC-139	BC-47	XXI1.1	XXI1.2	XXI.2	XXI	XX	VI
dove/Pak/Bal/WECD-121/2020		0.0011	0.0013	0.0015	0.0087	0.0037	0.0116	0.0110	0.0104	0.0112
dove/Pak/Bal/WECD-122/2020	0.0024		0.0012	0.0015	0.0090	0.0037	0.0118	0.0111	0.0106	0.0113
ch/Pak/Bal/BC-139/2020	0.0031	0.0018		0.0006	0.0085	0.0036	0.0121	0.0110	0.0107	0.0110
ch/Pak/Bal/BC-47/2020	0.0037	0.0024	0.0006		0.0085	0.0037	0.0121	0.0111	0.0108	0.0110
XXI.1.1 (n = 27)	0.1068	0.1075	0.1056	0.1059		0.0064	0.0095	0.0084	0.0062	0.0064
XXI.1.2 (n = 19)	0.0387	0.0377	0.0379	0.0386	0.0854		0.0095	0.0086	0.0079	0.0084
XXI.2 (n = 10)	0.1438	0.1440	0.1447	0.1456	0.1195	0.1301		0.0105	0.0079	0.0119
XXI (n = 7)	0.1332	0.1334	0.1323	0.1332	0.1021	0.1160	0.1375		0.0079	0.0093
XX (n = 17)	0.1201	0.1213	0.1220	0.1229	0.0962	0.1072	0.1209	0.1144		0.0061
VI (n = 25)	0.1201	0.1203	0.1193	0.1193	0.0899	0.1054	0.1316	0.1122	0.0856	

suggests viral replication. Noouzzaman *et al*. (2021) has recently reported that the viruses belonging to genotype XXI can cause clinical signs in chickens. Previously, the virulent strains of NDV of various genotypes (VII.2, XXI.1.1, and XXI.1.2) have been isolated from birds of the Columbidae family (Cross *et al*., 2013; Sabra *et al*., 2017) and have circulated in and adapted to these birds (Wajid *et al*., 2017). Wild birds have been considered a potential reservoir of virulent strains of NDV and AI viruses that may contaminate the environment through their dropping in and around commercial and backyard poultry farms and contribute to the transmission of these viruses (Si *et al*., 2013; Wajid *et al*., 2018). Infected wild birds can mix with free-ranging domestic poultry resulting in transmission through sharing of water points and feedlots. The minimal biosecurity measures and the lack of vaccination of free-ranging backyard chickens may increase the probability of transmission of viruses between Columbiformes and chickens. The pigeon-associated genotype VI has been reported to cause clinical disease in chickens and other non-Columbidae species kept in captivity (Sabra *et al*., 2017; Wajid *et al*., 2017). There is a relatively high contact rate between backyard poultry and wild birds in this region, increasing the risk of disease (Wajid *et al*., 2021).

In this study, four isolates have been phylogenetically classified into sub-genotype XXI.1.2. This is the first report of isolation of a genotype XXI.1.2 in Balochistan province, although these viruses have been isolated from other parts of the country between 2015 and 2022 (Wajid *et al*., 2016; Sabra *et al*., 2017). The nucleotides identify between the viruses isolated from collared doves and that from backyard chickens was high (99.6% to 99.9%) providing evidence of the existence of epidemiological links between the two species. Non-Columbidae species infected with pigeon-associated viruses often do not exhibit signs of clinical disease (Ren *et al*., 2017). Moreover, the XXI genotype is relatively new and has been discovered merely recently. XXI.1.1 of genotype XXI is composed of viruses isolated predominantly from pigeons in different countries including Pakistan, Egypt, Russia, Iran, Kazakhstan, Ukraine, Nigeria, and Bangladesh during 2005-2021,

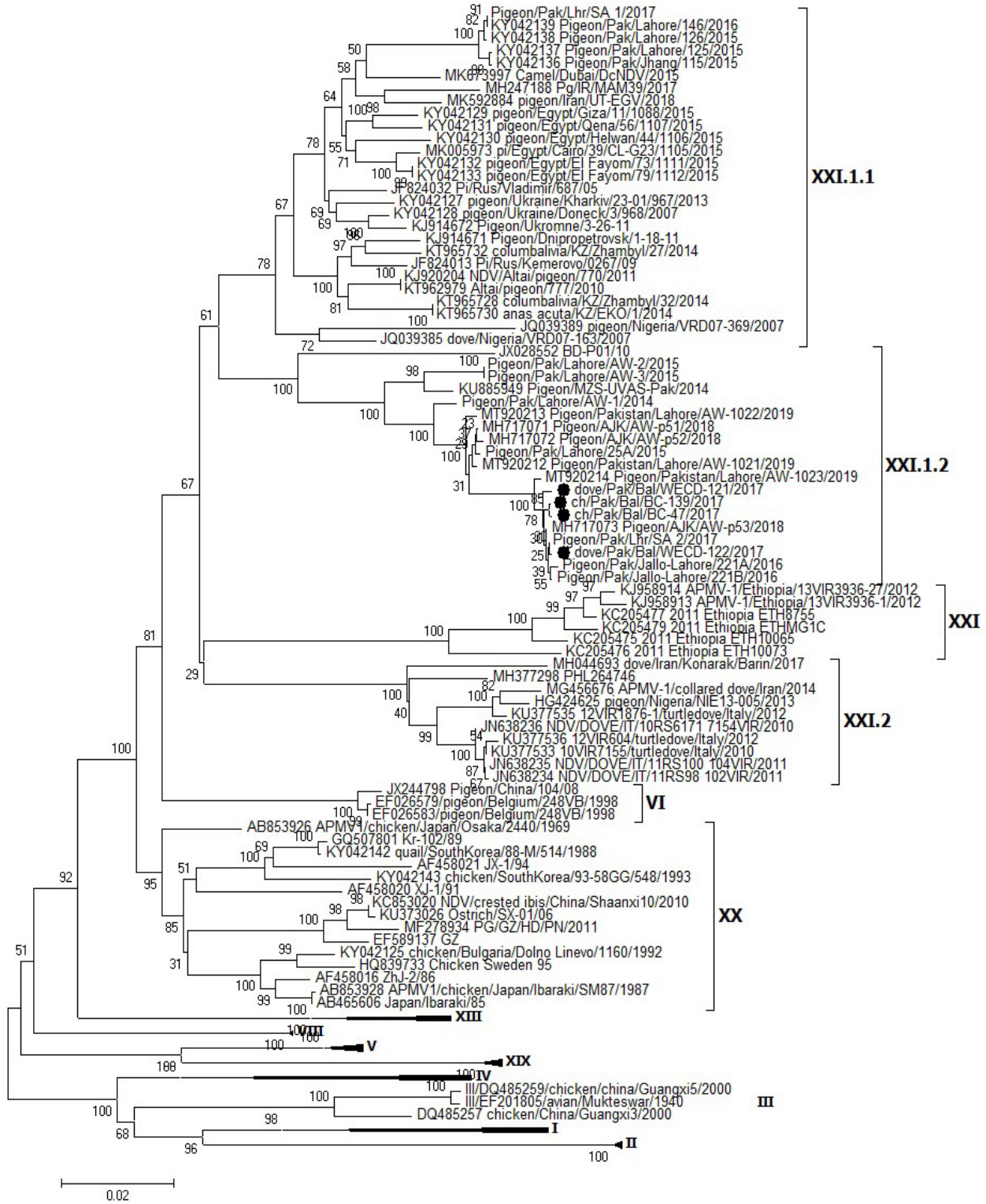

Fig. 1. Phylogenetic analysis based on full-length *F-gene* sequences of NDVs ($n = 71$). The tree was constructed using MEGA v6 employing the maximum-likelihood method and general time reversible model with 1000 bootstrap replicate. The black circles indicate the studied isolates.

while viruses belonging to sub-genotype XXI.1.2 have been isolated from pigeons in Pakistan only (Nasir *et al*., 2022). Viruses of genotype XXI were first identified in chicken in 2005, and it is unclear how and where these viruses were maintained. The immune poultry and feral birds may be the possible sources of maintenance of these viruses (Miller *et al*., 2015).

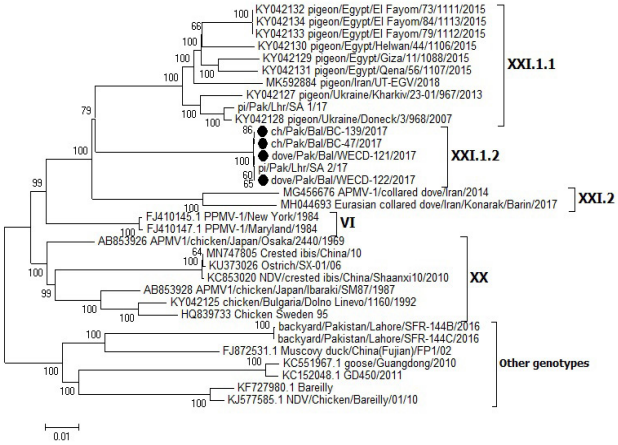

Fig. 2. Evolutionary analysis by maximum-likelihood method for the full-length genome sequences of NDVs (n = 47). The tree was constructed using MEGA v6 employing the maximum-likelihood method and general time reversible model with 1000 bootstrap replicate. The sequences characterized in this study are showed as black circles.

CONCLUSIONS

This study highlighted the possible role of feral collared doves in the dissemination of virulent NDV strains in backyard birds. The findings highlight the necessity of monitoring synanthropic and wild bird populations as part of NDV monitoring programs rather than just poultry populations. The isolation of pigeon-derived viruses from poultry emphasizes that the potential of these viruses to cause clinical disease in poultry must not be underestimated. Further studies are required since these recently identified viruses may pose a threat to poultry farming in Pakistan and other countries.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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