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Genomic and biological characterization of Pigeon Paramyxovirus Type-1 isolated from Indian pigeons: First report on the six nucleotide insertion in the non-coding region of nucleoprotein gene

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ABSTRACT

Genotypic analysis of Pigeon Paramyxovirus Type-1 (PPMV-1) virus, in corroboration with pathotyping, underpins the knowledge on the occurrence of PPMV-1 mediated Newcastle disease in poultry. In the present study, two PPMV-1 isolates, namely D167 and D168, obtained from feral pigeons of India in the year 2014 were analysed for their genotypic and pathotypic characteristics. Phylogenetic analysis of Fusion (F) gene grouped D167 and D168 under sub-genotypes VI.2.2.2. and VI.2.1.1.2.2. respectively. The separate positioning of these isolates within genotype VI is corroborated with the variation in restriction enzyme sites analysed through virtual restriction mapping. Analysis of FPCS region showed that both D167 and D168 possessed ^{112}RRQKRF^{117} amino-acid pattern. Intra-cerebral pathogenicity index (ICPI) of D167 and D168 was 1.57 and 1.3, and mean death time (MDT) was 48 and 70 hrs respectively, designating D168 as a mesogenic pathotype, and D167 as a velogenic pathotype. Histopathological analysis of proventriculus, intestine, lungs and spleen of chickens infected with D167 and D168 isolates through intramuscular (I/M), subcutaneous (S/C) and oculo-nasal/oral (O/N) routes of inoculation showed similar severity of tissue damage. This study shows that PPMV-1 isolates belonging to two different sub-genotypes without a common ancestor, can occur together in a particular geographical region. It also shows that PPMV-1 gains virulence in chickens, which needs to be addressed in order to avoid emergence of PPMV-1 as new velogenic variant causing Newcastle disease in chickens.

Keywords: Avian Orthoavulavirus 1, FPCS analysis, Genotyping, Newcastle disease, Pathotype, Pigeon Paramyxovirus

Pigeon Paramyxovirus–1 (PPMV-1) is a variant of Avian Orthoavulavirus–1 (AOaV-1) that causes Newcastle Disease (ND) in pigeons and is categorized under Genotype VI of AOaV-1 of *Orthoavulavirus* genus of Paramyxoviridae family. It has been speculated that multiple events of interspecies transmission of Genotype VI viruses of AOaV-1 between pigeons and chicken might have led to the origination of PPMV-1 in North East Africa (Ujvari *et al*. 2003) and in India (Mangat *et al*. 1988). Spill-over events of PPMV-1 from pigeons to poultry necessitated the need to study these viruses to understand their evolution dynamics.

PPMV-1 is an enveloped virus encompassing a single stranded, non-segmented, negative sense RNA genome. The complete genome of PPMV-1 consists of 15,192 nucleotides coding for six structural proteins (Millar and Emmerson 1988, Lamb and Parks 2007) namely nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and large polymerase (L). The 5' non-coding region of NP gene of PPMV-1 comprises an additional six nucleotides (6-nt), in contrast to the classical AOaV-1 genome (Phillips *et al*. 1998, Huang *et al.* 2004). The presence of additional 6-nt was observed between 1647 and 1648 nucleotide position of classical AOaV-1 genome (Huang *et al.* 2004, Shan *et al*. 2021). The amino acid sequence of Fusion protein cleavage site (FPCS) of PPMV-1 strains isolated so far consists either of ^{112}GRQKRF^{117} or ^{112}RRKKRF^{117} or ^{112}RRQKRF^{117}, characteristic of velogenic strains (Collins *et al*. 1994). However, their pathogenicity in chickens range from moderate to no virulence (Dortmans *et al.* 2010). In the recent past, PPMV-1 has been reported to gain virulence over passages in chicken or embryonated chicken egg (Kommers *et al*. 2003), which warrants the study of genotypic and pathotypic characteristics of PPMV-1 isolates. This will aid in understanding the evolution patterns of PPMV-1 and the possibilities of occurrence of PPMV-1 mediated ND in chickens. Therefore, in the present study, two PPMV-1 isolates, namely D167 and D168 isolated from pigeons in South India were subjected

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to genotypic and pathotypic characterization, which involved F gene based phylogenetic analysis, and intracerebral pathogenicity index (ICPI), mean death time (MDT), respectively along with histopathological analysis of proventriculus, intestine, lungs and spleen.

MATERIALS AND METHODS

Virus: Two PPMV-1 field isolates namely D167 and D168 obtained from pigeons of South India that had died showing typical neurological symptoms of ND were used in this study. These isolates were plaque purified in chicken embryo fibroblast (CEF) cells (Pushpa *et al.* 2009) and were propagated in allantoic cavity of 9-day old specific pathogen free (SPF) embryonated chicken egg (ECE) (OIE 2012) and the harvested amnio-allantoic fluid (AAF) was stored in -80°C until further use.

Extraction of total RNA and reverse transcription: Total RNA was extracted from D167 and D168 AAF using Trizol (Life Technologies, USA, Cat # 15596-026) as per manufacturer's instructions. Reverse transcription was done with 1 µg of total RNA using Verso cDNA synthesis kit (ThermoScript USA) following manufacturer's instructions.

Primers: Amplification of the complete gene sequence of F gene of both D167 and D168 PPMV-1 isolates were done in two overlapping fragments, F1 and F2, using previously published primers (Awu *et al*. 2015) as provided in Table 1.

PCR amplification and nucleotide sequencing: The complete F gene and 5' non-coding region of NP gene of D167 and D168 were amplified using Primestar high fidelity enzyme mix (Cat# R010A, Takara Clontech, USA) as per manufacturer's instructions. In brief, the reaction mixture comprised of 25 µl of 5× PrimeStar master mix, 0.5 µM of each primer, and 10 ng of cDNA template in a final reaction volume of 50 µl. The reaction cycle involved initial denaturation at 98°C / 30 s followed by 35 cycles of denaturation at 98°C / 10 s, optimum annealing temperature / 10 s and extension at 72°C / 60 s with a final extension at 72°C /5 min. The details of the primers used are provided in Table 1. The resultant PCR products were separated on agarose gel and were purified using Nucleospin Gel and PCR cleanup kit (Cat#740609.50, Machery-Nagel, Germany) and was subjected to nucleotide sequencing using BDT v3.0 cycle sequencing kit on ABI 3730xl Genetic Analyzer. The nucleotide sequences of the complete F gene, and the 5' non-coding region of NP gene of both D167 and D168 were aligned contiguously using overlapping nucleotides using BioEdit software version 7.1 (Hall 1999).

Construction of phylogenetic tree: The complete nucleotide sequence of F gene of D167 and D168 were aligned and was subjected to ClustalW multiple sequence alignment along with "pilot dataset" made available for genotype identification of new isolates (Dimitrov *et al.* 2019) comprising a total of 137 F gene sequences with three representative sequences of each genotype / sub-genotype of Class I and Class II AOaV-1 and phylogenetic tree was constructed using maximum-likelihood algorithm (Tamura and Nei 1993) with 1000 bootstrap replicates using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al.* 2021).

Restriction enzyme analysis and recombinant detection: A virtual restriction map based on F gene, for the presence of Hinf1, BstO1 and Rsa1 restriction enzymes (RE), was created for D167 and D168 along with sequences of genotype VI with the help of online restriction mapper (https://restrictionmapper.org).

Biological characterization of D167 and D168: Biological characterization of D167 and D168 involved estimation of MDT and ICPI. MDT was carried out in 9-day old SPF ECE and ICPI was done in day-old SPF chicks as per international standards of OIE (2004), approved by Institutional Animal Ethical Committee (IAEC) (3028/ DFBS/B/2014).

Analysis of NP gene: The nucleotide sequence of 5' non-coding region of NP gene of both D167 and D168 was subjected to ClustalW multiple sequence alignment using BioEdit sequence alignment software (Hall 1999) along with 20 gene sequences of AOaV-1 retrieved from Genbank and was analyzed for 6-nt insertion between nucleotide positions 1647 and 1648 of the AOaV-1 complete genome. NP gene sequences of strains or isolates belonging to genotypes I to VII and IX of AOaV-1 were used for analysis. NP gene sequences of Genotype VIII and X, XI, XII and XIV to XXI were not available in GenBank.

Histopathological analysis: The PPMV-1 isolates D167 and D168 were inoculated into ten-day-old SPF chicks through different routes like I/M, S/C and O/N (n=10). Each group was inoculated with 10^6 ELD_{50} per 0.1 ml. Five chicks per route were sacrificed on tenth and twenty first day post inoculation respectively. The tissue samples of proventriculus, intestine, lungs and spleen were collected and fixed in 10% formalin for 48 h and was processed for histopathological analysis (Awu *et al*. 2015).

Table 1. Details of primers used

Primer name	Gene covered	Primer sequence	Ta (°C)	Nucleotide position based on	Product size (bp)
F1f	F	AAGCACCCGAGAAGATCCCTG	58	3768 – 4909	1162
F1r	F	AGAGCTACACTGCCGATAATG			
F2f	F	TATGCCCAAGGACAAAGAGG	58	4753 – 6460	1936
F2r	F	CCATGTGTTCTTTGCTTCTCT			
NPf	5' non-coding region of NP	GCATCAGTCTTGGATAAGGGAAC	58	1133 – 1953	820
NPr		CAATGACAGTTCCACTGGTCTCA			

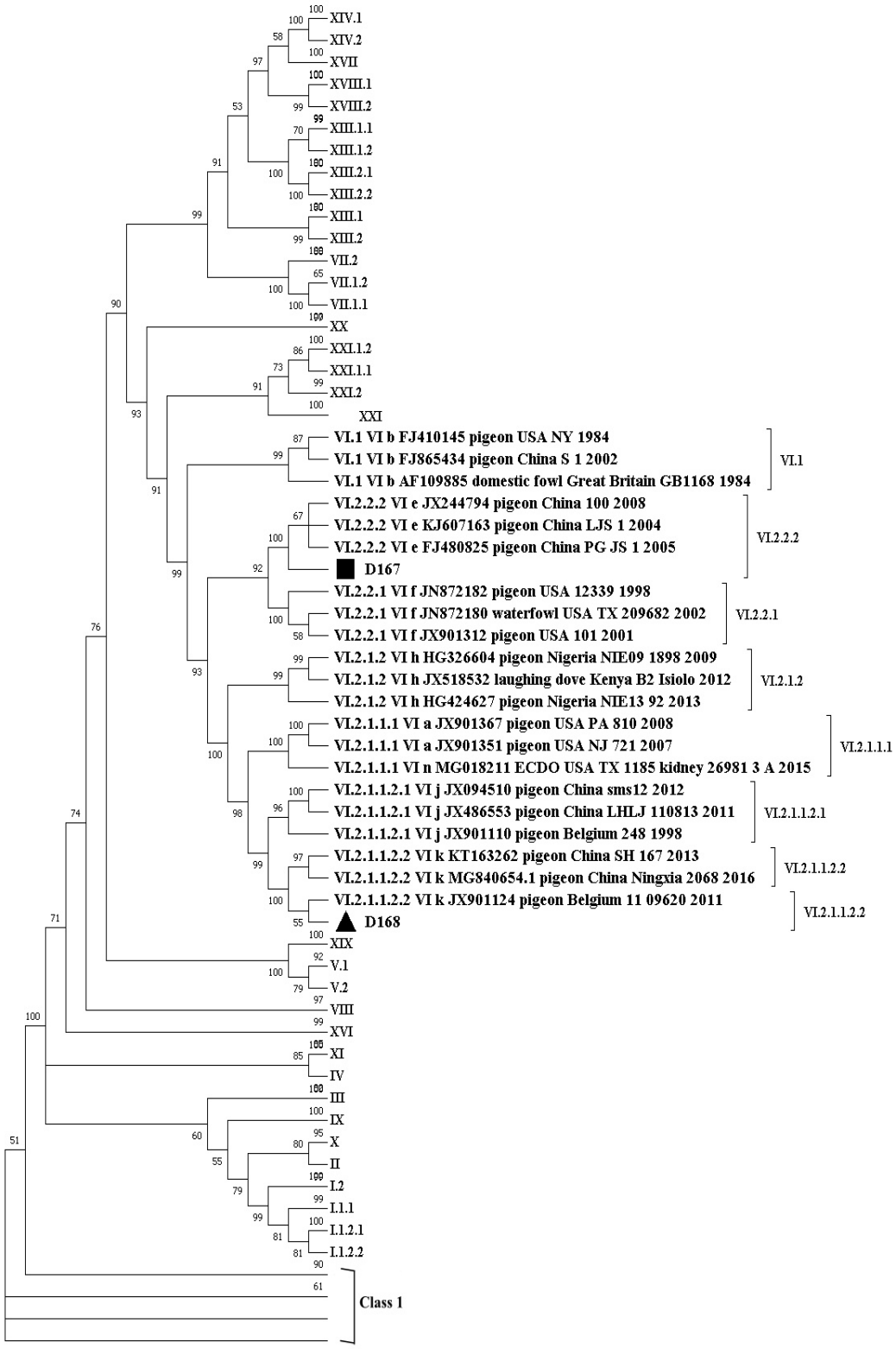

Fig. 1. Molecular Phylogenetic analysis by Maximum Likelihood Method – Fusion (F) gene. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 139 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1802 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

RESULTS AND DISCUSSION

Immaterial of the pathotype, pigeons affected with PPMV-1 are asymptomatic with the ability to shed virus through oral, tracheal, and cloacal routes, thus posing a threat of ND outbreak in poultry population (Afonso 2021). To prevent the possibilities of occurrence of PPMV-1 mediated ND in poultry, vaccination of pigeons, and monitoring the occurrence of PPMV-1 in pigeons are vital. For effective design of vaccines and diagnostic tools, genotypic and pathotypic data of PPMV-1 isolated from specific geographical regions would be helpful. In this study, two PPMV-1 isolates obtained from feral pigeons of tropical climatic region, that showed torticollis were analyzed for genotypic and pathotypic characteristics.

Phylogenetic classification of AOaV-1 using complete F gene sequences has been considered authentic in identification of genotypes of AOaV-1 (Dimitrov *et al*. 2019). In this study, the complete nucleotide sequence of F gene of D167 (Accession no. KX710209) and D168 (Accession no. KX710210) was sequenced and submitted to GenBank. Phylogenetic analysis of F gene of the PPMV-1 isolates D167 and D168 along with the "pilot dataset" of AOaV-1 genotypes (Dimitrov *et al.* 2019), positioned D167 under sub-genotype VI.2.2.2, and D168 under sub-genotype VI.2.1.1.2.2 of genotype VI (Fig. 1). In the updated genotype classification, it has been observed that the isolates under the sub-genotypes VI.2.2.2 and VI.2.1.1.2.2 consisted predominantly of isolates obtained from China (Dimitrov *et al.* 2019).

Upon analysis of the amino acid sequence of F gene, it was observed that both D167 and D168 isolates possessed ^{112}RRQKRF^{117} at the FPCS region identical to that of virulent AOaV-1. Further, amino acid Isoleucine (I) at 121 and Serine (S) at 124 of F gene was observed in both D167 and D168, characteristic of velogenic AOaV-1 and certain PPMV-1 isolates reported earlier (Naveen *et al.* 2013). In addition to this, it was also observed that amino acid Threonine (T) at 108 and Serine (S) at 132 of F gene were present in both the isolates, which have been reported to be unique features of PPMV-1 isolates (Naveen *et al.* 2013). All PPMV-1 isolates originated in India between 1998 and 2002 were found to have Glutamic acid (E) at 104 and Threonine (T) at 107 (Naveen *et al.* 2013). However, both D167 and D168 possessed Glycine (G) at 104 and 'S' at 107, suggestive of evolutionary divergence (Kumanan *et al*. 2002, Naveen *et al.* 2013).

MDT value of D167 and D168 was 48 and 70 h respectively; and ICPI value of D167 and D168 was 1.57 and 1.3, respectively. On the basis of the standard MDT values prescribed to classify the AOaV-1 into velogenic – less than 60 h, mesogenic – more than 90 h and lentogenic – more than 90 h (OIE 2012), D167 is classified as velogenic, and D168 is classified as mesogenic. Several studies indicate that majority of PPMV-1 isolates have been reported to have mesogenic MDT (Alexander and Swayne 1998, Qin *et al.* 2008, Khan *et al.* 2010, Munir *et al.* 2012, Wang *et al.* 2013, Uthrakumar *et al.* 2014, Dey *et al.* 2014). However, in the present study MDT of D168 correlates with previous work while but D167 showed variation. Further, on the basis of ICPI standard values prescribed to classify AOaV-1 as velogenic – >1.5, mesogenic – 0.7 – 1.5 and ≤0.7 – lentogenic pathotypes (Allan *et al.* 1978), both D167 and D168 can be categorized under mesogenic pathotype. PPMV-1 isolates have been reported to possess a fairly low ICPI value ranging between 0.69 – 1.4 (Meulemans *et al*. 2002). However, in the present study, D167 exhibits an ICPI value more than the usual range for PPMV-1 isolates. Such variation in MDT and ICPI values for PPMV-1 isolates have been reported post passage in chickens (Seal *et al.* 2000, Kommers *et al.* 2003, Saif *et al*. 2008). This further affirms the perils of PPMV-1 gaining virulence due to spill over and spillback of the virus between pigeon and chicken.

Experimental inoculation of D167 and D168 in ten-day old chicks through different routes of inoculation (I/M, S/C, O/N) showed notable histopathological changes in proventriculus, intestine, lungs and spleen without any marked clinical signs and necropsy lesions. Experimental infection through all the routes of inoculation for both D167 and D168 showed mononuclear cell infiltration in mucosa and necrosis of glands in proventriculus; necrotic enteritis or haemorrhagic enteritis in intestine; congestion in lungs; and congestion, lymphoid depletion and germinal centre formation in spleen (Supplementary Fig. 1). Moreover, it was observed that the severity of tissue changes did not vary with the route of inoculation, in contrast to the previous studies showing variation in severity based on the route of inoculation (Dai *et al*. 2014, El-Morshidy *et al*. 2021). Further, the absence of overt clinical signs and gross lesions in SPF chicks experimentally infected with D167 and D168, indicates that PPMV-1 can cause sub-clinical infection in chickens that shed AOaV-1 through natural routes, thereby acting as carriers.

The virtual restriction map analysis of D167 and D168 were found to have variations in RE sites. RE sites for *HinfI* was observed at position 829, 837 and 1354 in D167, whereas in D168, one more additional site at position 1018 was observed. RE sites for *BstO1* in D167 were found in 856, 1070, 1214 and 1432 positions whereas for D168, one more additional site at 1114 position was observed. RE sites for *RsaI* were found to be at 1041 and 1579 position in D167, whereas in D168 an additional site at 1114 was observed. Hence, it has been concluded that the separate positioning of D167 and D168 within Genotype VI of AOaV-1 could be corroborated to the variations in the RE sites (Czegledi *et al*. 2006).

In the identification for the presence of 6-nt insertion in the NP gene that is specific for PPMV-1 isolates, it was observed that D167 and D168 possessed an additional 6-nt (TCTCAA) in the 5' non-coding region of NP gene (Fig. 2). The additional 6-nt insertion was present between the 1647 and 1648 nucleotide position of Lasota, Komarov, Texas GB, B1, VG/GA, Mukteswar and Herts33 genomes;

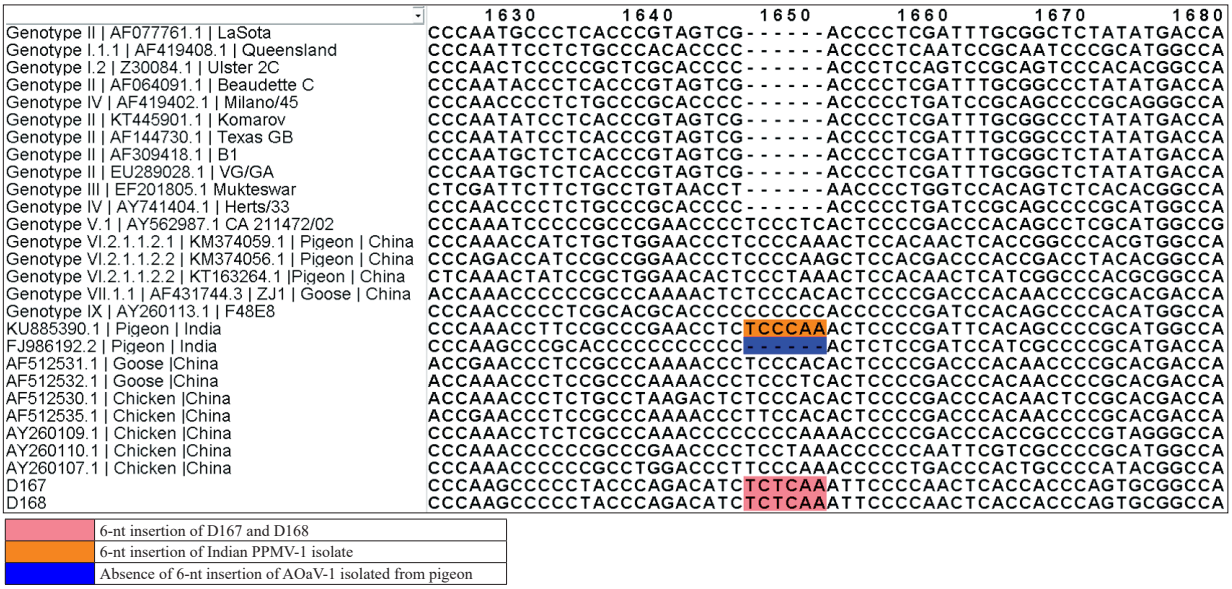

Fig. 2. Presence of 6-nt insertion sequence in 5' non-coding region of NP gene of D167 and D168.

Table 2. Presence and similarity of 6-nt insertion in the NP gene in different avian hosts and geographical location

Chicken		Pigeon		Geese	
China	Africa	China	India	Australia	China
CCCCAA	CCCCAT	CCCCAA	TCCCAC	TCTAAA	TCCCTC
TCCCAC	CCCCAC	CCCCAC	TCCCAA		TCCCAC
TCCCAA					
TTCCAC					
TCCTAA					

Same colours across columns indicate similarity of sequences.

and between the 1592 and 1593 nucleotide position of Queensland, Ulster 2C, Beaudette C and Milano 45 genomes. The nucleotide sequence of the additional 6-nt in the NP gene of AOaV-1 isolates obtained from chicken, pigeon and goose in China, India, Africa and Australia (Huang *et al*. 2004, Wang *et al*. 2015, Awu *et al*. 2015, Qiu *et al*. 2017, Shan *et al*. 2021) is given in Table 2. Upon perusal of the 6-nt insertion, it was observed that the same sequences were present in PPMV-1 isolates obtained from chicken and pigeon in India, China and Africa, reaffirming the fact that spill over and spillback of PPMV-1 occurs between avian hosts.

In India, only two complete genome sequences of AOaV-1 isolated from pigeon were available in GenBank. Of which, the PPMV-1 isolate obtained from pigeon possessed additional 6-nt of TCCCAA (Ganar *et al*. 2017); whereas the AOaV-1 isolate NDV/2K3 obtained from pigeon (Accession no. FJ986192) did not possess the additional 6-nt (Fig. 2), thus confirming that NDV/2K3 is not PPMV-1 (Tirumurugaan *et al*. 2011). Therefore, to the best of our knowledge, this is the first study in India reporting the presence of 6-nt insertion in the 5' non-coding region of NP gene of PPMV-1 isolated from pigeons.

In conclusion, two PPMV-1 isolates namely D167 and D168 obtained from pigeons in South India, were subjected to genotypic and pathotypic characterizations. Phylogenetic analysis grouped D167 sub-genotype VI.2.2.2, and D168 under sub-genotype VI.2.1.1.2.2 of genotype VI, along with other mesogenic PPMV-1 isolates. FPCS amino acid deduction showed ^{112}RRQKRF^{117} sequence similar to that of virulent AOaV-1; and the 5' non-coding region of NP gene possessed additional 6-nt insertion characteristic of PPMV-1 isolates. Biological studies including MDT and ICPI for D167 and D168 were slightly inclined towards velogenic AOaV-1 suggesting gain of virulence as a result of passage in chicken eggs. However, histopathological studies indicated that both D167 and D168 did not cause severe tissue damage. Hence, it is speculated that PPMV-1 isolates despite showing genomic and biological characteristics of virulent AOaV-1 did not cause evident clinical signs or severe gross or histopathological lesions. However, the probabilities of PPMV-1 gaining virulence in chicken over a period of time and the possibilities of recombination events due to spill over and spillback of PPMV-1 between chicken and pigeon needs to be studied extensively to understand the evolution patterns of PPMV-1.

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