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Genetic characterization of pigeon- origin avian avulavirus-1 reveals unique substitutions in F and HN proteins

Deepthi Balam[1]*, Ratnamma Doddamane[2], Ramani Pushpa Rn[3], Shrikrishna Isloor[2], Veeregowda Bm[2]

[1] Department of Veterinary Microbiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Garividi, India; [2] Department of Veterinary Microbiology, Veterinary College, Karnataka Veterinary, Animal and Fisheries Science University, Bangalore, India; [3] Department of Veterinary Microbiology, NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram, India.

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Abstract

Since decades, Newcastle disease (ND) has become endemic in the poultry population of the Indian subcontinent. ND is a highly contagious disease of poultry and other avian species. However, the genetic nature of ND viruses circulating in the rock pigeons is unraveled. The present investigation is a part of Newcastle disease virus (NDV) surveillance in wild birds. Two velogenic NDV strains could be isolated from apparently healthy rock pigeons, thus establishing the status of carrier/reservoir host. The fusion protein cleavage site in the fusion protein has multiple basic amino acid (RRRKRF) motifs similar to velogenic isolates. Phylogenetic analysis based on complete fusion gene sequences confirmed that the isolates belong to NDV sub genotype XIII 2.2. Further analysis revealed several amino acid substitutions in the hypervariable region, heptad repeat regions and neutralizing epitopes of the fusion protein and heptad repeat regions and antigenic sites of the hemagglutinin-neuraminidase (HN) protein that are critical for fusion. A unique D170A substitution in the neutralizing epitope is identified that is critical for structure and function of the fusion protein. Mutations within the virulence determinants including fusion (F) and HN, elucidate continuous evolution of the viruses among the rock pigeons. Accidental spillover of these mutated viruses into commercial poultry operations may result in disease outbreaks with economic breakdown.

Introduction

Newcastle disease (ND) in chicken and other birds is caused by virulent strains of avian avulavirus-1 (AAvV-1), popularly known as Newcastle disease virus (NDV). The ND outbreaks in fully susceptible birds may result in 100% flock mortality.[1] During epizootics, the disease often spread rapidly among flocks resulting in serious economic losses in terms of reduced production as well as restraints in trade and export. As a consequence, World Organization for Animal Health has classified ND as a "notifiable" disease.[2] Extensive disease outbreaks, broad host spectrum, multiple NDV genotypes and inappropriate vaccination protocols made ND, one of the major limiting factors for the poultry industry, especially in developing countries like India. The AAvV-1 belongs to genus *Avian orthoavulavirus 1* within the subfamily *Avulavirinae* of the family *Paramyxoviridae*.[3] The genome of NDV is a negative sense; single- stranded RNA with 15.20 Kb in size organized into six genes each encoding for six structural proteins, namely, *Nucleoprotein (NP), Phosphoprotein (P), Matrix (M) protein, F protein, HN protein and large polymerase (L) protein*, as well as two non-structural proteins, *V* and *W*. Even though several molecular determinants were attributed to NDV pathogenicity, fusion protein cleavage site (FPCS) in the fusion protein is one of the well accepted pathogenic determinants in chicken.[4] Besides the FPCS sequence, mean death time (MDT) in 9-day old embryonated chicken eggs and intracerebral pathogenicity index (ICPI) in day- old chicks are the other pathogenicity determinants to assess the virulence of NDV strains.[5] Based upon the severity of the disease, NDV strains are classified into three categories: lentogenic (apathogenic), mesogenic (intermediate pathogenic) and velogenic (highly pathogenic) strains. Further, based on the full - length fusion gene sequences, NDV has been classified into two major classes, I and II. Evolutionary diversity within class I NDV is low, and, mostly includes viruses of low virulence, except, for a report of one class I virulent virus. Class II NDV are highly diverse and includes

***Correspondence:**

Deepthi Balam. MVSc, PhD
Department of Veterinary Microbiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Garividi, India
E-mail: drdeepthib@gmail.com

a range of non- virulent to virulent viruses, classified into 21 genotypes with multiple sub genotypes.[6]

Domestic and wild birds belonging to 241 species from 27 orders of the avian family are reported to be susceptible to ND.[7] Majority of the birds also serve as a reservoir host for both velogenic and lentogenic Avian Paramyxo viruses. Under favourable circumstances, reservoir hosts may transmit pathogens to the domestic poultry. [2]

In India, reports that discuss the reservoir nature of birds for NDV are scanty. Hence, a study was carried out to ascertain that apparently healthy rock pigeons (*Columba livia*) could act as reservoir hosts for NDV.

Materials and Methods

Cloacal swabs were collected from apparently healthy rock pigeons (n = 45) raised in lofts by pigeon fanciers in Visakhapatnam, East Godavari and West Godavari districts of Andhra Pradesh, India. Virus isolation was attempted from the samples using 9- day old embryonated chicken eggs. Presence of NDV was confirmed by hemagglutination activity as well as hemagglutination inhibition (HI) test. Lasota, a lentogenic vaccine strain (IVPM, Ranipet, India) was used as antigen (10^5 EID_{50} per vial) for preparation of hyper-immune serum (HI test) as well as positive control during virus isolation and reverse transcriptase polymerase chain reaction (RT-PCR). Each sample was passaged blindly thrice before considering it as negative. Virulence of the NDV isolates was determined by MDT in nine- day old embryonated chicken eggs and ICPI in day-old chicks.[5]All the experiments carried out in embryonated chicken eggs, day-old chicks and three-week-old chicken were performed duly following the 'Guide for the Care and Use of Laboratory Animals' that was approved by Institutional Animal Ethics Committee, NTR College of Veterinary college, Gannavaram, India.

The NDV isolates identified by positive HI test were further confirmed by RT-PCR. Total RNA was extracted from the amnioallantoic fluid employing TRIzol™ reagent (Genei, Bangalore, India) following manufacturer's instructions. First strand complimentary DNA (cDNA) was synthesized using random hexamers and iScript™ cDNA synthesis kit (BioRad, Hercules, USA) according to manufacturer's protocol. Complimentary DNA thus synthesized was used for identification of NDV by targeting partial fusion gene as well as for genotyping by amplification of complete fusion and HN genes. Amplification of partial and complete fusion genes was done using previously published primers, while complete HN gene was amplified by using primers designed for this study (Table 1). Reaction conditions for amplification of complete HN gene were as follows: 98.00 ˚C for 3 min followed by 35 three-step cycles of 98.00 ˚C for 10 sec, 65.00 ˚C for 30 sec and 72.00 ˚C for 2 min; then 72.00 ˚C for 10 min. The amplified PCR products were analyzed using 1.00% agarose gel containing ethidium bromide and were visualized under Ultraviolet. The PCR amplicons obtained in RT-PCR were sequenced at M/s Barcode Biosciences (Bangalore, India).

Chromatograms obtained from the sequencing firm were analyzed and edited for any errors using a software, Codon code aligner (version 4.2.5; CodonCode Corp., Centerville, USA). Identity of the sequences was confirmed by BLAST analysis within the non- redundant nucleotide databases. The F and HN gene sequence data of the isolates were subjected to blast analysis with the help of NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with the NDV sequences available in GenBank®. Multiple alignment of the coding regions of each of the two genes were performed using the CLUSTAL W Multiple Sequence Alignment Program (UCD, Dublin, Ireland) algorithm in MEGA Software (version 11.0; Biodesign Institute, Tempe, USA).[10] Phylogenetic tree was inferred to localize the pigeon isolates amongst representative class II reference strains according to the maximum-likelihood method based on the general time-reversible model as implemented in MEGA Software (Table 2). Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum composite likelihood approach, and then selecting the topology with superior

Table 1. Details of primers used in the study.

Primer name		5' to 3' sequence	Product size	References
FPCS	F	GCAGCTGCAGGGATTGTGGT	365 bp	8
	R	TCTTTGAGCAGGAGGATGTTG		
Complete fusion gene (1662 bp)	F1- F	CACTAAGATAGAGAAGAG GCACACC	1058 bp	9
	F1-R	TTATACAGTCCAATTCTCGCGCC		
	F2- F	AAAGAGGCATGTGCAAAAGCCCC	929 bp	
	F2-R	GTGTAGTGAGTGCACCTTCAGTCT		
	F3-F	GGGAGCCTAAATAATATGCGCGCC	815 bp	
	F3-R	GCG CCA TGT ATT CTT TGC TTC		
Complete HN gene (1716 bp)	HN ext-F	AGAACG GTCAAAGGAGCCAC	1860 bp	Designed for this study
	HN ext-R	GGTGCAACACCTTCCTTCCA		
	HN int-F	TCAATACTTGGCACTCGGTG		
	HN int- R	TTAGGTGGAACAGTCAGCAC		

log likelihood value. The maximum likelihood method based on the General Time Reversible model with a discrete gamma distribution (five categories [+G]) was utilized for all the trees. The trees were generated by using the NJ algorithm, and alignments were bootstrapped 1,000 times. All positions containing gaps and missing data were eliminated. The evolutionary relationship between the two NDV isolates obtained in the study and 49 representative strains belonging to 21 genotypes of class II NDV were estimated by calculating the number of base differences per site from averaging over all sequence pairs between groups using MEGA Software. Details of the accession numbers used for phylogenetic analysis were mentioned in Table 2.

Table 2. AAvV-1 reference sequences used in this study for phylogenetic analysis and evolutionary distance estimation.

Isolates	Accession No.
NDV_isolate/BAREILLY/2013	KF727980.1
AAvV-1_strain_CHICKEN/SWEDEN/97	GU585905.1
NDV_strain/BAREILLY/2011	HQ589257.1
AAvV-1 Ostrich/South Africa/45445-3/1995	JN942034.1
NDV_strain/CHICKEN/4132-20/BURUNDI/2008	FJ772494.1
NDV_strain/CHICKEN/IRAN/EMM/7/2011	JQ267579.1
APMV-1/ Chicken/Bangaladesh/bd-C50/2010	KY905318.1
NDV_isolate/COCKATOO/INDIA/7847/1982	JN942041.1
APMV-1/Chicken/ Bangladesh/KP242/2012	KU936220.1
NDV strain chicken/Pakistan/NDV/UDL8/2011	JQ517285.1
NDV strain SD/5/04/Go fusion protein	DQ682445.1
NDV isolate NDV/Chicken/Nagpur/04/11	KX372708.1
NDV isolate ndv52/Sarsa	KM056350.1
AAvV-1_isolate/AAVV-116/GODHRA/03/2013	KM056344.1
APMV-1/ migratory bird/Bangladesh/P57/2011	KU936227.1
NDV/Chicken/ Kamrup/07/14	KX345397.1
NDV isolate Pandu fusion protein and hemagglutinin-neuraminidase genes	KT734766.1
NDV_strain/CHICKEN/RANCHI/01/2014	KR072665.1
AAvV-1/ Peru/1918-03/603/2008	JN800306.1
AAVV-1_isolate_2007/Mali/ML038/07	JF966389.1
NDV strain chicken/Nigeria/NIE09-2087/2009	HF969155.1
NDV/Cockatoo/Indonesia/14698/90	AY562985.1
NDV Chicken / U.S (CA)/1083 (Fontana)/ 72	AY562988.1
AAvV-1 isolate Pigeon/Pak/Lahore/AW-2/2015	KU862298.1
AAvV-1_isolate_ZhJ-3/97	FJ766529.1
NDV/ Japan/Ibaraki/85	AB465606.1
AAvV-1_strain_AF2240-I	JX012096.1
NDV isolate QH4	FJ51919.1
AAvV-1/Chicken/Dominican Republic/867-2/2008	JX186997.1
APMV-1/chicken/Ca/2098/71,	JQ247691.1
AAvV-1 isolate Cormorant/Florida/41105/2012	KC433530.1
AAvV-1_strain/ANHINGA/US_(Fl)/44083/93	AY562986.1
AAvV-1_isolate_XJ-2/97	AF458011.1
AAvV-1_strain/MG/1992	HQ266603.1
AAvV-1_strain/ITALIEN	EU293914.1
NDV-2/chicken/Namakkal/Tamil Nadu,	GU187941.1
AAvV-1_isolate_2K3/Chennai/Tamil_Nadu/2000	FJ986192.1
AAvV-1_isolate/JS/9/05/Go	FJ430160.1
AAvV-1_strain_Mukteswar	EF201805.1
AAvV-1_strain/BLACKBIRD/CHINA/08	KC934169.1
NDV strain ZJ/1/86/Ch, complete genome	FJ436303.1
AAvV-1_isolate/ I-2	AY935499.1
NDV_isolate/COCKATOO/INDIA/7847/1982	JN942041.1
NDV virus strain northern pintail/US(OH)/87-486/1987	GQ288378.1
AAvV-1_strain_Mallard/US(OH)/04-411/2004	GQ288377.1
AAvV-1_strain/LASOTA	AF077761.1
NDV turkey/USA/VGGA/89	AY289002.1
AAvV-1_strain_B1	AF309418.1
AAvV-1 GD450/2011	JN627508.1

Results

Of the 45 cloacal swab samples, two samples collected from the same farm in Tanuku, West Godavari district, resulted in peri-occipital hemorrhages and diffuse congestion in the inoculated dead embryos during the second passage; and were identified as NDV based on HI test as well as RT-PCR (PGN1 and PGN2). The total isolation rate was 4.44% (2/45). Since both the NDV isolates obtained in this study were found to be similar as proven by pathogenicity tests, and due to financial shortfall, only PGN1 isolate (NDV/ Pigeon (PGN1)/ 2018/ Tanuku/Andhra Pradesh) was further characterized and submitted for complete F and HN gene sequencing.

The PGN1 isolate was found to be mesogenic NDV strain based on MDT and ICPI (MDT- 60h and ICPI- 1.45, each experiment carried out twice). The amino acid motif at the cleavage site of the fusion protein (FPCS) was found to be ^{112}R-R-R-K-R-F^{117} with more than three basic amino acids and phenylalanine at position 117,[5] suggestive of velogenic NDV strain. The velogenic FPCS motif was inconsistent with the determined MDT and ICPI values, as these indices indicate an intermediate nature of virulence (mesogenic isolate).

Full-length F and HN gene sequences were available in GenBank® under the accession numbers, MT 362716 and MT909567 respectively. Phylogenetic analysis based on the complete fusion gene, clustered the PGN1 isolate with other viruses of class II, sub- genotype XIII 2.2. The evolutionary distance estimates based on deduced amino acid sequences of F and HN proteins, suggests that the PGN1 isolate showed a divergence of 10.50 - 13.35% and 11.33 - 13.90% respectively from the widely used NDV vaccine strains. Among the vaccine strains, the PGN1 isolate shared greatest identity with F protein (10.50%) and HN protein (11.30%) of NDV- Mukteshwar, a mesogenic strain.

Comparison of the predicted amino acid sequences revealed several substitutions within the functional domains of fusion and HN proteins. Hypervariable region of the fusion protein of PGN1 isolate resembled Mukteshwar strain at few residues (Table 3). V22I motif usually present in sub genotype XIII 2.2 was not detected in PGN1. In addition, N30S motif was found which is also observed in Mukteshwar strain. Unique D170A substitution was identified among the neutralizing epitopes that are critical for structure and function of fusion protein. Amino acid differences in the heptad repeat regions and other critical domains of the fusion protein are depicted in Tables 4 and 5. While, two transmembrane domains located between amino acids 499 - 525 and 501 - 523 manifested significant differences, the six N-glycosylation sites at positions ^{85}NRT^{87}, ^{191}NNT^{193}, ^{366}NTS^{368}, ^{447}NIS^{449}, ^{471}NNS^{473} and ^{541}NNT^{543} were unaltered.

Table 4. Amino acid differences in the heptad repeat regions of the fusion protein of PGN1

HRa (143-186)	HRb (268-299)	HRc (471-500)	HRd (81-102)
K145N*	T270A	N479D*	D82E*
D170A	G271A	E482A	
-	N272Y	R486S*	-
-	L282I	K494R*	-
-	T288N	-	-
-	S291T	-	-
-	N294S	-	-

Amino acid residue substitutions at the indicated positions when PGN1 is compared to LaSota.
* indicates similarity with Mukteshwar strain at these residues.

Table 5. Amino acid differences in the other domains of fusion protein of PGN1

Amino acids	Substitutions
103 - 142	E104G, T107S, I121V, G124S†
187 - 267	F190L, K192N†, Q195R, A203T, N231T†, K232Q†, N258S* R312K†, T341S, D342N, I386L†, K395E*, M396I, V402A†,
300 - 470	N403D†, K421R, Q422H†, N425S*, G430D†, V442A†, Q451L†, D452E†, I457V†
501 - 553	I509T*, V513I, F514S, I516A, I520V

* indicates unique substitutions not found in reference/vaccine strains and † indicates similarity with Mukteshwar strain at these residues.

The HN protein of PGN1 isolate had an open reading frame of 571 aminoacids. Among the three transmembrane domains located in the HN gene, PGN1 isolate exhibited differences in two domains (24 - 47 and 25 - 45), while the sialic acid binding site, glycosylation sites and cysteine residues remained the same.

Discussion

Since its first identification a century ago, ND still remains a constant threat to the poultry population including chicken and other wild birds. Although several vaccine strains are being used, complete protection against the disease could not be achieved and outbreaks occur

Table 3. Amino acid differences in the hyper variable region of the fusion proteins of PGN1 isolate in comparison with vaccine strains

Strains	4	8	9	11	13	14	16	17	20	28	29	30	32	69	82
LaSota	R	K	N	A	M	M	T	I	A	P	A	N	I	L	D
R_2B	●	●	●	T	●	●	●	V	●	●	●	●	●	●	●
B_1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Komarov	●	●	●	T	●	●	●	V	●	●	●	●	●	●	●
Mukteshwar	●	**R**	I	V	**L**	●	●	●	T	**L**	**T**	**S**	**L**	M	**E**
PGN1	K	**R**	V	I	**L**	G	I	T	M	**L**	**T**	**S**	**L**	I	**E**

* Amino acids that match the consensus (LaSota strain) exactly are denoted by ●. The bolded residues were similar in PGN1 and Mukteshwar.

resulting in heavy economic losses. In the present investigation, two NDV strains (PGN1 and PGN2) could be isolated from apparently healthy pigeons. PGN1 was further characterized for its virulence as partial velogeic/ mesogenic, based on the fusion gene sequence and the biological characterization parameters, i.e., MDT and ICPI. In agreement to our findings, Liu *et al.*[11] reported that, of the 14 pigeon- origin NDV strains isolated from sick pigeons, 13 were proved to be mesogenic based on MDT and ICPI, though multiple basic amino acid residues were found in FPCS. In contrary, other studies reported isolation of velogenic NDV from suspected ND outbreaks in wild pigeons.[12,13]

Previous studies emphasized characterization of NDV strains based on gene sequences of F and HN.[14] We also in this study, characterized full- length F and HN genes as well as hypervariable region of the fusion gene to determine the genotype of the isolate and to understand the phylogenetic relationship of the isolate to the representative strains of NDV across the globe. Complete F and HN gene sequences of the PGN1 isolate were submitted to GenBank® and are available under accession numbers, MT362716 and MT909567 respectively. Based on complete fusion gene, PGN1 isolate clustered with sub genotype XIII 2.2 of class II viruses (Fig.1). Since, the first isolation of the genotype XIII ancestral strain from a

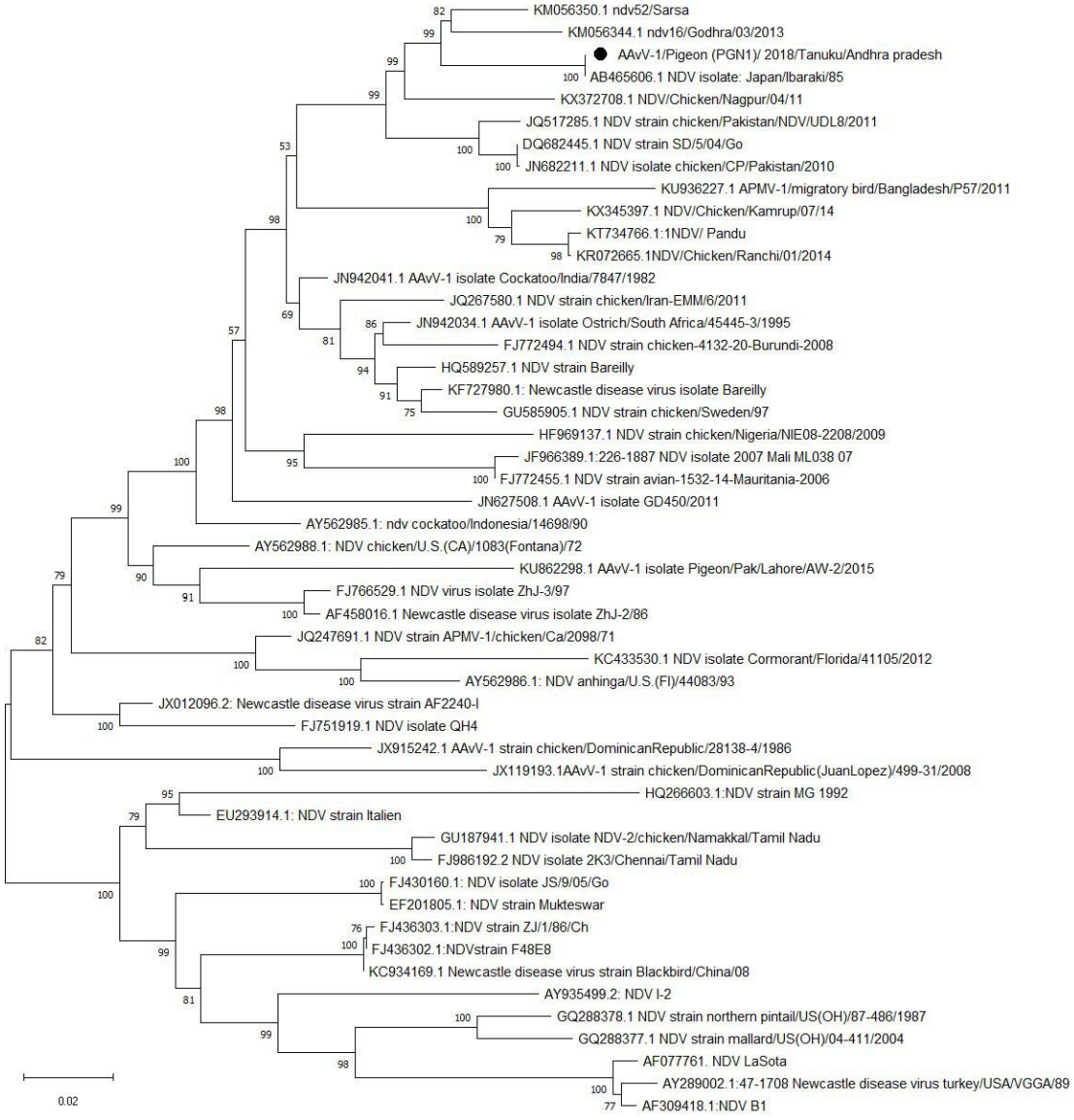

Fig. 1. The evolutionary history was inferred by using the maximum-likelihood method (Tamura-Nei model). The tree with the highest log likelihood (-16,429.20) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 50 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. There were a total of 1,663 positions in the final dataset.

cockatoo, several other strains evolved and are classified under sub- genotypes 1.1, 1.2, 2.1 and 2.2.[15,16] Among the genotype XIII 2.2 strains, PGN1 shares close identity with chicken- origin NDV strains isolated during ND outbreaks from different parts of India (KX372708.1- NDV/ Chicken/Nagpur/04/11; KM056350.1- NDV52/ Sarsa). This suggests circulation of the very same virus in different avian species demonstrating explicit cross species transmission of pathogens. In a similar study conducted by us in spot billed pelicans that migrate every year to a wild life sanctuary in Telineelapuram, coastal Andhra Pradesh, India, we could isolate virus of the same sub genotype III 2.2.[17]

The HN proteins of various lengths (571, 577, 603 amino acids) had been reported previously from different strains of NDV, the PGN1 isolate had an open reading frame that encoded for 571 amino acids, a common feature of most of the velogenic NDV's.[18]

The third NDV panzootic was due to pigeon- adapted variants affecting primarily pigeons and poultry occasionally.[19] Pigeon-origin NDV's are genetically diverse with variable virulence and can result in enteric, visceral and neurological form of disease in the infected birds and has potential to cause outbreaks in poultry.[20] Due to lack of proper geographical segregation, these birds have adequate access to naïve or susceptible poultry substantiating enhanced biosecurity within poultry houses where these rock pigeons usually abound.

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Conflict of interest

The authors state that there is no conflict of interest.

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