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Simultaneous detection of velogenic Newcastle disease virus of genotype XIII 2.2 from spot-billed pelican and backyard chicken: implications to the viral maintenance and spread

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Summary. – Despite the widespread occurence of Newcastle disease virus (NDV) in different avian species, there has been scanty reports on genetic characterization of NDV strains from wild birds in India. During 2017–18, a total of forty eight cloacal swab samples were collected from apparently healthy migratory birds (painted storks, n = 32 and spot-billed pelicans, n = 16) at the Telineelapuram bird sanctuary of Andhra Pradesh, India. NDV was isolated from a spot-billed pelican (NDV/Pelican/Telineelapuram/2018) which is genetically identical to that isolated from a naturally infected backyard chicken flock (NDV/Chicken/SKLM-1/2018). The isolates are found to be velogenic based on mean death time, intracerebral pathogenicity index and the putative fusion protein cleavage site ([112]R-R-R-K-R-F[117]). Phylogenetic analysis based on full-length fusion gene classified the isolates into genotype XIII, sub-genotype 2.2, however these isolates demonstrated multiple amino acid substitutions in the critical domains of F and HN proteins. The pelican strain (MIG-9) was tested for its pathogenic and transmission potential in three-week-old broiler chickens and the isolate proved to be highly virulent to chickens. To the best of our knowledge, this is the first evidence for the role of spot-billed pelicans in the maintenance of virulent NDV and its transmission to chickens in India. This study further highlights the role of wild birds in NDV transmission and the need for enhanced biosecurity in commercial poultry operations.

Keywords: Newcastle disease virus; Pelecanus philippensis; chicken; transmission; pathogenicity; India

Introduction

Newcastle disease (ND) is a devastating infectious disease of poultry causing serious economic losses to the poultry industry as well as the farmers worldwide. ND is caused by avian paramyxovirus 1 (APMV-1) or Newcastle disease virus (NDV) belonging to the genus *Avian orthoavulavirus* (AOAV-1) within the subfamily *Avulavirinae* of the family *Paramyxoviridae* (ICTV, 2019). The disease is characterized by rapid onset of respiratory and nervous symptoms in susceptible species of birds including chickens, pigeons, and turkeys. Wide range of domestic and wild birds are susceptible to avian paramyxovirus infections worldwide. Birds of 241 species from 27 orders are reported to be susceptible to ND (Kaleta and Baldauf, 1988).

NDV is an enveloped virus with helical symmetry, and carries negative sense single-stranded RNA genome.

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Abbreviations: AAvV = avian avula virus; FPCS = fusion protein cleavage site; HI = hemagglutination-inhibition test; HN = hemagglutinin neuraminidase; ICPI = intracerebral pathogenicity index; MDT = mean death time; ND = Newcastle disease; NDV = ND virus

The genome has been reported to possess six genes in the order of nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin neuraminidase (HN) and large polymerase (L) and translated into eight proteins. Of the eight proteins encoded by the genome, F and HN are the key glycoproteins associated with the envelope (Miller and Koch, 2013). Fusion glycoprotein is responsible for fusion with, and penetration through the cell membrane. Fusion protein encompassing fusion protein cleavage site (FPCS) is the key virulence factor that determines the NDV pathotype (OIE, 2012). HN glycoprotein is responsible for attachment of the virus to host cell receptors and also possess receptor destroying property. Heptad repeat domains of F and HN proteins promote protein-protein interactions and mediate fusion promotion (Stone-Hulslander and Morrison, 1999). Further, based on the full-length fusion gene sequences, NDV has been classified into two major classes, I and II. All the avirulent isolates were grouped under class I, which includes a single genotype with three sub genotypes. Class II contains most of the virulent viruses circulating worldwide, classified into at least 21 genotypes with multiple sub genotypes (Dimitrov *et al.,* 2019) and this diversity continues to increase as the surveillance studies improve.

Virus spillovers between domestic chickens and wild birds have been the novel research area. Avian influenza studies highlighted the role of reservoir host in perpetuation of a pathogen and subsequent spillover into domesticated population resulting in severe form of outbreaks (Swayne *et al.,* 2014). Likewise, virulent and avirulent NDV of several genotypes were often identified in wild birds like water fowl, and have been speculated to play a role in the spread of the virus (Snoeck *et al.,* 2013). Role of the spot-billed pelicans or grey pelicans (*Pelecanus philippensis*) and painted storks (*Mycteria leucocephala*) that breed only in Peninsular India, Sri Lanka and Cambodia, in the epidemiology of NDV is unknown; furthermore, transmission potential of pelican-origin NDV in chickens have not been evaluated previously. Therefore, the present investigation aimed to evaluate the pelican-origin and chicken-origin NDV isolates for their pathogenicity and transmissibility to naïve chickens.

Materials and Methods

Ethical statement. Samples from the migratory birds were collected under the supervision of forest department personnel following all the guidelines and regulations. All the animal experimental protocols performed in this study are approved by Institutional animal ethics committee of NTR college of veterinary science, Gannavaram, Andhra Pradesh (Approval No. 5/IAEC/NTRCVSC/2018).

Sampling procedures. Telineelapuram bird reserve, a home to painted storks and spot-billed pelicans located in Srikaku-

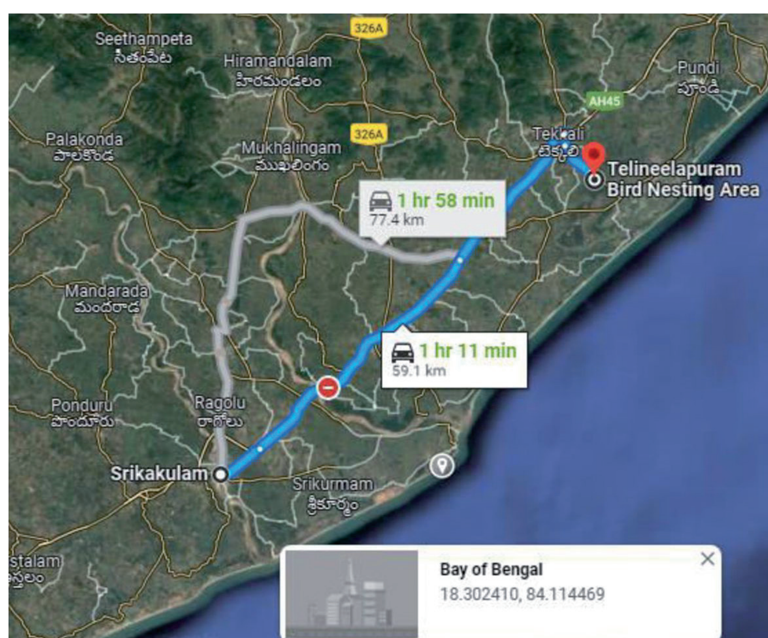

Fig. 1

Location of sample collection sites
Telineelapuram bird sanctuary and Srikakulam in the coastal belt of Andhra Pradesh, India.

lam district of Andhra Pradesh, India is considered one of the major wintering areas within the central Asian flyway that extends from Siberia to West Asia, India and Maldives (Fig. 1). Painted storks and spot-billed pelicans reach here during the month of September or October and stay here till May or June. Coastal belt including Srikakulam district is a prime poultry hub in Andhra Pradesh and is endemic to various poultry diseases including ND. A total of forty eight cloacal swab samples (painted storks, n = 32 and spot-billed pelicans, n = 16) were collected during February, 2018, from clinically healthy adult birds that were in a non-flying stage in the enclosures. Cloacal swabs were collected in phosphate buffered saline (PBS) supplemented with an antibiotic mixture (2000 unit/ml penicillin G, 200 mg/ml streptomycin and 4 mg/ml amphotericin B) and frozen at -20°C until used in virus isolation and processed as per standard procedures (OIE, 2012). The NDV/Chicken/SKLM-1 strain was isolated from backyard poultry flocks that were reported to have died of ND during December, 2017 in Srikakulam district of Andhra Pradesh, India.

Virus isolation and biological characterization. Cloacal swabs were processed as 10% homogenates in 500 µl of PBS supplemented with antibiotics and inoculated into 9-day-old embryonated chicken eggs via the allantoic route. Each sample was passaged blindly thrice before considering it as negative. Presence of NDV was confirmed by detecting hemagglutination and hemagglutination activities of the amnioallantoic fluid (AAF) collected from the embryos. LaSota, a lentogenic vaccine strain (IVPM, Ranipet, India) was used as antigen (10^5 EID_{50} per vial) for raising hyper-immune serum and as positive control during virus isolation and RT-PCR. Virulence of the NDV isolates was determined by mean death time (MDT) in nine-day-old embryonated chicken eggs and intracerebral pathogenicity index (ICPI) in one-day-old SPF chicks (OIE, 2012). NDV pathotypes were classified based on MDT, as follows: velogenic, if death occurs within 60 h; mesogenic, 60–90 h; and lentogenic, more than 90 h. Based on ICPI, velogenic viruses give ICPI indices that approach the maximum score of 2.0, whereas lentogenic strains give values close to 0.0.

Detection of NDV by RT- PCR. The NDV isolates positive in hemagglutination-inhibition test (HI) were further confirmed by reverse transcription PCR. The AAF harvested during the third passage was used for RNA isolation employing TRIsol reagent (Genei, India) following manufacturer's instructions. First strand cDNA was synthesized using random primer and iScript™ cDNA synthesis kit (Biorad, USA). The isolates were confirmed as NDV by polymerase chain reaction (PCR) using primers targeting FPCS (Nantha Kumar *et al.,* 2000). Full-length fusion gene was amplified using previously published primers (Gowthaman *et al*., 2018), while HN gene was amplified using primers designed for this study (Supplementary Table S1).

Gene sequence and phylogenetic analysis. The amplified PCR products were sequenced by Sanger sequencing with the help of commercial sequencing centre (Barcode Biosciences, India). The 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 avian paramyxovirus type – 1 sequences available in GenBank (Supplementary Table S2). Multiple alignment of the coding regions of each of the two genes were performed using the clustalW algorithm in MEGAX (Kumar *et al*., 2018). Phylogenetic tree was inferred to localize MIG-9 and SKLM-1 amongst representative class II reference strains according to the maximum-likelihood method based on the general time-reversible model as implemented in MEGA X software.

Pathogenicity assay in three-weeks old chickens. A total number of thirty six, three-weeks old white Leghorn chickens (*Gallus gallus domesticus*) were obtained from M/s Sreenivasa hatcheries, Vijayawada, India. All the chicks were housed in an isolated, well-ventilated room and provided with balanced ration and water *ad libitum* throughout the experiment. All biosecurity measures were taken care off. Once the birds were acclimatised, they were confirmed to be free of NDV antibodies by HI test before the start of the experiment. Of the thirty six birds, thirty were split into 3 groups, group 1 (control, n = 10), group 2 (MIG-9, n = 10) and group 3 (SKLM-1, n = 10). At day 0, birds of both groups were inoculated with 0.5 ml (10^3EID_{50}) of the respective NDV isolate *via* subcutaneous route. Control group was placed in separate facility and sham inoculated with 0.5 ml PBS on the same day. Virus transmission to naïve, uninfected chickens was assessed by placing three birds in a separate cage at 2 feet distance from the cages of infected groups on the next day. Infected and sentinel birds were observed regularly for clinical signs and mortality.

Results

Virus isolation and biological characterization

Of the forty eight cloacal swab samples collected from the migratory birds, two samples from spot-billed pelicans and one from the painted stork showed perio-ccipital haemorrhages and diffuse congestion in the inoculated dead embryos; however, only one sample from spot- billed pelican was found positive for NDV. The total isolation rate was 0.021% (1/48) and the isolate was referred as NDV/Pelican/ Telineelapuram/2018 (MIG-9). The SKLM-1 and MIG-9 isolates confirmed by HI were further confirmed by RT-PCR targeting partial fusion gene encompassing FPCS. Besides, the MDT and ICPI values also classified the isolates as velogenic (Table 1).

Genetic characterization

The amplified F and HN gene sequences of SKLM-1 and MIG-9 isolates were submitted to GenBank and are avai-

Table 1. Pathogenicity features of the chicken and pelican NDV isolates

Isolate	MDT (h)	ICPI	FPCS	HA titre	HI
AAvV/Chicken/SKLM-1/2018	45	1.80	^{113}RRKR ↓F^{117}	2^6	positive
AAvV/Pelican/ Telineelapuram/2018	48	1.85	^{113}RRKR ↓F^{117}	2^5	positive

AAvV = avian avula virus.

lable under Acc. Nos. MT036311(SKLM-1/F); MZ270546 (SKLM-1/HN); MN901912 (MIG-9/F); MT909566 (MIG-9/HN). The isolates are velogenic strains with ^{112}R-R-R-K-R-F^{117} motif at F protein cleavage site. Topology of the phylogenetic tree constructed based on full-length F gene revealed that the two isolates were 100% identical and clustered closely with the strains of genotype XIII sub-genotype 2.2 of class II viruses (Fig. 2). Based on the fusion gene sequences, the isolates under study share 99.2% identity with NDV isolate 410/16A (GenBank ID MF422129), 98.3% identity with NDV isolate 96–15 (GenBank ID MF422125) and 97.8% with NDV isolate D162 (GenBank ID KX242342), which are also placed under the same subgenotype. The deduced amino acid sequence of the fusion protein showed a divergence of 0.8% with sub-genotype XIII 2.2 isolates, while the divergence with current vaccine strains ranged from 10–12.9%. The deduced amino acid sequences of the HN protein showed a divergence of 0.3–1.57% with sub-genotype XIII 2.2 isolates and 11.3–14.5% with current vaccine strains.

Analysis of fusion and HN proteins

Fusion gene was found to have a coding sequence of 1662 nucleotides coding for 553 amino acids. Amino acid sequence at the FPCS cleavage site motif ^{112}R-R-R-K-R-F^{117} has more than two pairs of basic amino acids and a Phenylalanine in the N terminus of the F1 protein (Table 1) typical for velogenic isolates. Table 2 shows the amino acid substitutions within the fusion peptide, heptad repeat regions (HRa, HRb, HRc and HRd) and transmembrane domains of the study isolates in comparison to the vaccine strains. Of the eight transmembrane domains in the fusion protein, six domains located at positions 15–25, 118–131, 120–128, 266–269, 429–432 and 499–525 were unaltered, while several substitutions were noted in the domains, 14–27 and 501–523. Six potential N-glycosylation sites (Asn-X-Ser/Thr or N-X-S/T, where X represents any amino acid except aspartic acid or proline) at positions ^{85}NRT^{87,} ^{191}NNT^{193,} ^{366}NTS^{368,} ^{447}NIS^{449,} ^{471}NNS$^{473 \text{ and }}$ ^{541}NNT^{543} were unaltered in the study isolates. Amino acid residues within the hyper variable region and neutralizing epitopes of the study isolates were compared with the vaccine strains (Table 3). A unique M14G substitution is identified in the hyper variable region of the study isolates. Interestingly, amino acid residues at positions 8, 9, 13, 28, 29 and 31 were similar to that in Mukteshwar strain, and to other vaccine strains at positions, 10, 25, 26, 27 and 30. Neutralizing epitope sequences critical for structure and function of the fusion protein were similar to that of the vaccine strains.

HN gene was found to have a coding sequence of 1716 nucleotides coding for 571 amino acids. Three transmembrane domains at positions 24–47, 25–45 and 557–563, a sialic acid binding site at position 234-239 and 13 Cysteine (C) residues at positions 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531 and 542 were recognised. The Cysteine (C) residue at position 123 was also located in R2B and Mukteshwar vaccine strains, while it has been replaced by Tryptophan (W) in LaSota, Komarov and B1. Six glycosylation sites were recognised at positions 119, 341, 433, 481, 508 and 538. As compared to the vaccine strains, the study isolates have amino acid substitutions at positions G75S, N77G, V81I and I84V in heptad repeat region A and T101S and T102I substitutions in heptad repeat region B.

Five neutralizing epitopes have been recognised in the HN protein of which, two epitopes, site 23 (193–201) and site 1 and 14 (345–355) remained unaltered, while three epitopes, site 12 (494), site 2 and 12 (513–521) and site 2 (569) presented numerous substitutions (Table 4). Three amino acids at positions, 401E, 416R and 526Y characterized by functional triarginyl cluster essential for receptor binding and neuraminidase activity are conserved in the study isolates. HN protein motifs responsible for hemagglutinating activity, ^{234}NRKSCSV/I/L^{240}, ^{314}FPVYGGL/V/M^{320} and ^{399}GAEGRIL/V/I^{405} remained unaltered in the study isolates.

Pathogenicity assay in three-week old chickens

Infection of three-week-old chicken resulted in virulent ND with 100% mortality. Clinical signs, gross and histopathological lesions induced by either of the isolates appeared similar and indicated that they caused systemic infection in the infected chickens and replicated quickly in multiple tissues. Birds exhibited clinical disease (depression, reluctancy to move, open mouthed breathing, mild conjunctivitis, ruffled feathers and greenish diarr-

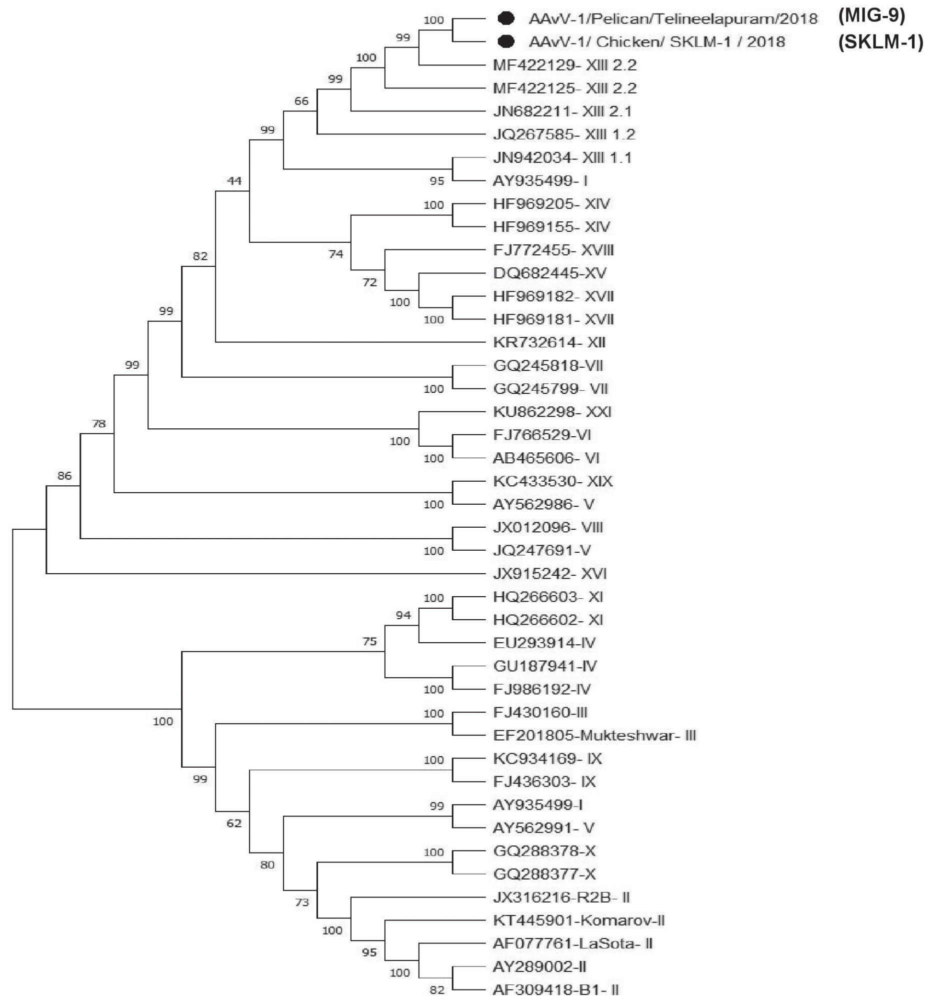

Fig. 2

Evolutionary analysis based on nucleotide sequences of the fusion gene of viruses representing NDV class II by Maximum likelihood method

The evolutionary history was inferred by using the Maximum likelihood method and General Time Reversible model. The tree with the highest log likelihood (-14950.65) 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 the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 43 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

hoea) at 2 day post infection which became more severe until death at 4 or 5 day post infection. Also, neurological signs like ataxia, twitching, head tremors were observed hours before death. Birds exhibiting severe clinical signs were euthanised to perform necropsy. Gross lesions were found widespread throughout different tissues. Most frequent necropsy findings include haemorrhages in the proventriculus and caecal tonsils, swollen kidneys, tracheal haemorrhages and splenomegaly associated with mottling (Fig. 3). The sentinel birds also started exhibiting clinical signs similar to that shown by directly inoculated birds 5 days post exposure progressing to severe clinical signs by 7 days post exposure. Hundred percent mortality was observed in all the infected and sentinel birds. Control birds appeared clinically normal throughout the observation period.

Histological lesions were analysed in the tissues of infected, sentinel and control birds. No major differences were noticed in the birds infected with either of the viruses and in the sentinel birds. In spleen, lesions varied from vacuolar changes to necrosis of the lymphoid tissue in the white pulp characterized by lymphocytolysis and lymphoid depletion. Heart revealed pericarditis and myocarditis characterized by degeneration of myocar-

Table 2. Comparison of amino acid substitutions within the functional domains of fusion protein sequences of the chicken and pelican NDV strains with common vaccine strains

Virus strains	Fusion peptide (117-141)			HRa (143-185)	HRb (268-299)				HRc (471-500)				HRd (81-102)		Transmembrane domains										
															14-27					(501-521)					
	117	121	124	145	272	282	288	291	294	479	482	486	494	82	14	16	17	20	22	509	513	514	516	518	520
LaSota	L	I	G	K	N	L	T	S	N	N	E	R	K	D	M	T	I	A	V	I	V	F	I	S	I
R_2B	F	-	-	-	-	-	-	-	-	G	-	S	-	-	-	-	V	-	-	V	-	L	M	-	-
B_1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Komarov	F	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	V	-	-	-	-	-	-	-	-
Mukteshwar	F	-	-	N	-	-	-	-	-	D	-	S	R	E	-	-	-	T	A	V	-	L	M	-	-
AAvV/Chicken/ SKLM/2018	F	V	S	N	Y	I	N	T	S	D	A	S	R	E	G	I	T	M	I	A	I	S	A	G	V
AAvV/Pelican/ Telineelapuram/ 2018	F	V	S	N	Y	I	N	T	S	D	A	S	R	E	G	I	T	M	I	A	I	S	A	G	V

" – " no change in amino acid compared to that of LaSota.

Table 3. Comparison of amino acid substitutions in fusion protein in the hypervariable region and neutralizing epitopes of the chicken and pelican NDV strains with common vaccine strains

Virus strains	Hypervariable region																	Neutralizing epitopes		
	4	**10**	**11**	**13**	**14**	**16**	**17**	**20**	**22**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**69**	**72,74,75,78,79**	**157-171**	**343**
LaSota	R	P	A	M	M	T	I	A	V	C	I	C	P	A	N	S	L	DEAKA	SIAATNEAVHEVT-DG	L
R_2B	-	-	T	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-
B_1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Komarov	-	-	T	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-
Mukteshwar	-	-	V	L	-	-	-	T	A	Y	V	R	L	T	S	-	M	-	-	-
AAvV/Chicken/ SKLM-1/2018	K	-	I	L	G	I	T	M	I	-	-	-	L	T	-	-	I	-	-	-
AAvV/Pelican/ Telineelapuram/ 2018	K	-	I	L	G	I	T	M	I	-	-	-	L	T	-	-	I	-	-	-

" – " no change in amino acid compared to that of LaSota.

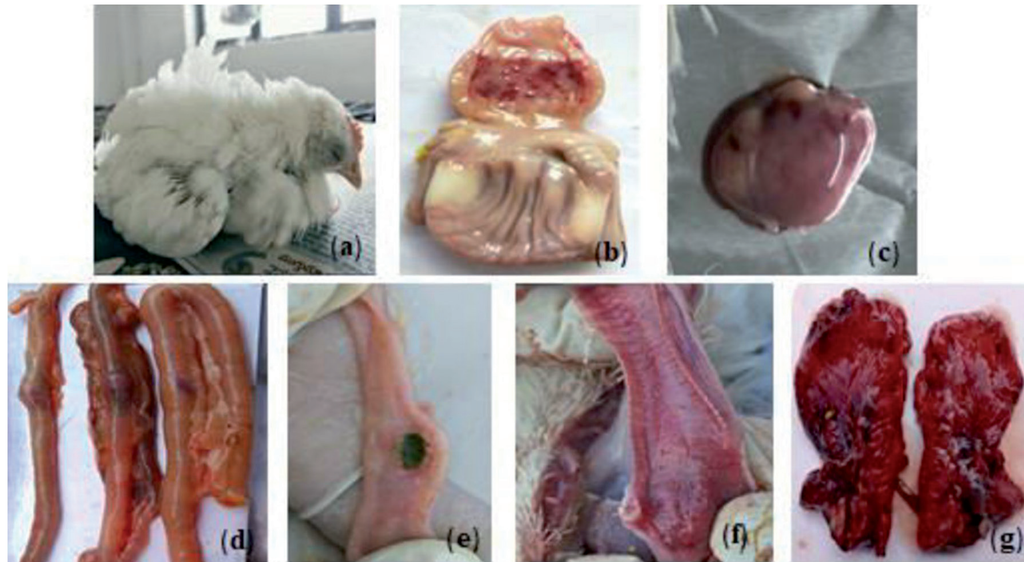

Fig. 3.

Clinical signs and gross lesions observed after infection of chicks
(a) Moribund chick; **(b)** hemorrhages at the tips of proventricular glands; **(c)** spleen showing mottling and focal necrotic areas; **(d)** and **(e)** caecal tonsil necrosis with typical button ulcers; **(f)** tracheal hemorrhages; **(g)** hyperemic lungs.

dium, infiltration of heterophils, fibrin, fibroblasts and mononuclear cells in the pericardium and myocardium. In the small intestines, lesions varied from catarrhal enteritis characterized by severe goblet cell hyperplasia to fusion, necrosis of villi and desquamation of epithelium into lumen. Lesions observed in the trachea during acute phase of respiratory disease were haemorrhages and vacuolar changes in the tracheal mucosa, syncytia formation and denudation of tracheal epithelium. Lungs revealed severe congestion and consolidation of parenchyma, denudation and exudation of bronchiolar epithelium. In the kidneys, lesions included nephrosis characterized by degeneration to severe tubular necrosis and interstitial nephritis (Fig. 4). No specific lesions were observed in the control group.

Discussion

Southeast Asia not only hosts the largest population of commercial and backyard poultry in the world (Roohani *et al.,* 2015) but is also recognized as a unique biodiversity hotspot with one of the richest and diverse avifauna. Coastal wetlands of southeast Asia represent major wintering and staging sites for millions of water bird species that migrate from Siberia, Russia, Germany and Australia.

Table 4. Comparison of amino acid substitutions within the functional domains of HN protein of the chicken and pelican NDV strains with common vaccine strains

Virus strains	HRa (74-89)				HRb		Neutralizing epitopes				
							Site 12	Site 2 and 12 (513-521)			Site 2
	75	77	81	84	101	102	494	514	519	521	569
LaSota	G	N	V	I	T	T	G	I	S	S	D
Komarov	-	-	-	-	-	-	-	-	-	-	-
R2B	-	-	-	-	S	I	-	-	-	-	-
B1	-	-	-	-	-	-	-	-	-	-	-
Mukteshwar	-	-	-	-	S	I	D	-	-	-	-
AAvV/Chicken/ SKLM-1/2018	S	G	I	V	S	I	D	V	A	R	V
AAvV/Pelican/ Telineelapuram/2018	S	G	I	V	S	I	D	V	A	R	V

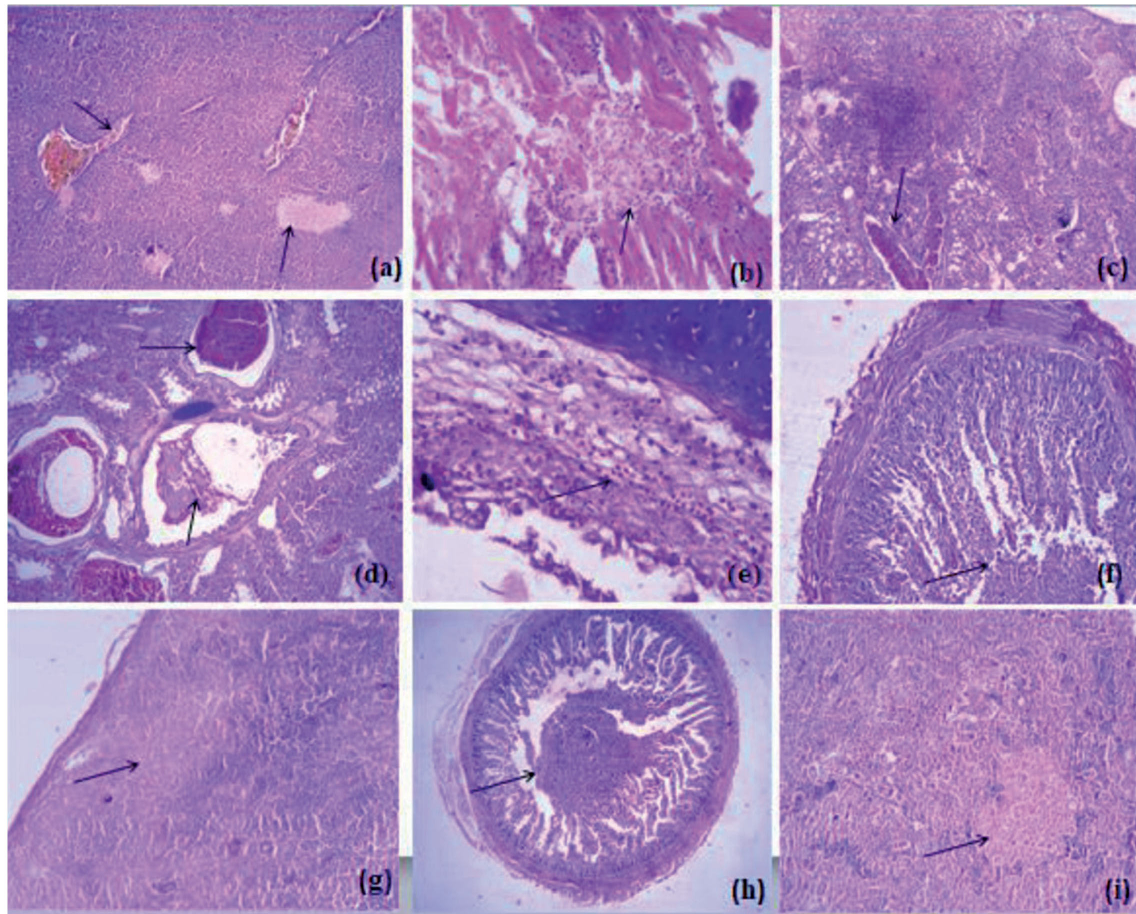

Fig. 4

Histopathological lesions in H&E stained tissue sections of experimentally infected chicks

(a) Liver – pockets of necrosis and congestion (4x); **(b)** heart – myocardial degeneration with infiltration of mononuclear cells (40x); **(c)** and **(d)** lungs – tertiary bronchiolar epithelial hypertrophy, congestion and exudation in the lumen (4x); **(e)** trachea – necrosis of tracheal epithelium, haemorrhage and infiltration of macrophages, lymphocytes, plasma cells and fibrin (40x); **(f)** intestine – necrosis of intestinal villi and denudation into the lumen (10x); **(g)** spleen – lymphoid depletion and diffuse necrosis (4x); **(h)** jejunum – denudation of epithelium **(i)** into lumen (4x); (i) kidney – severe tubular necrosis (10x).

The long coastal line and rivers in India also attract several species of migratory birds which present a unique opportunity to study mechanisms of viral maintenance and spread in this region. However, limited information is available regarding the potential role of these avian species in the dissemination of pathogens. Monitoring of birds for infectious agents such as NDV in their natural habitats and in areas that pose a risk of transmission between domestic poultry and wild birds will increase the knowledge of epidemiology, ecology and genetic relationships of such viruses. Hence, wild bird sanctuary located at Telineelapuram in Andhra Pradesh state was chosen for the present study and samples were collected from migratory birds like painted storks and spot-billed pelicans. This sanctuary has encroached into the human habitations and there is a fair chance of spillover and exchange of pathogens between the migratory birds and backyard poultry.

Phylogenetic analysis based on the full-length sequences of fusion and HN genes indicated a close genetic relationship between the chicken- and pelican origin NDV and grouped the isolates under genotype XIII subgenotype 2.2. The study isolates are more closely related to NDV isolate 410/16A (GenBank ID MF422129) and NDV isolate 96-15 (GenBank ID MF422125). Phylogenetically, genotype II, IV, VII and XIII strains of NDV are circulating in Southeast Asia (Esmaelizad *et al*., 2016). Several isolations of genotype XIII viruses were previously reported from other countries like Sri Lanka, Pakistan, Iran, Tanzania, and Russia mostly from chickens (Gowthaman *et al*., 2018; Zhu *et al*., 2016). Infection with genotype XIII 2.2 NDV in an emu flock was reported previously (Gowtha-

man *et al*., 2016). Among the genotype XIII 2.2 isolates, a variety of FPCS motifs were identified and this provides additional grounds for continued evolution of genotype XIII viruses in the country (Desingu *et al*., 2016). FPCS motif of the present study isolates, ^{112}RRRKR↓F^{117} resembles that of genotype VIf NDV isolates responsible for the third ND panzootic (Kwon *et al*., 2003) and this gives strength to the theory that the genotype XIII viruses might have re-emerged consequent to repeated passages in different bird species crossing geographical barriers, thereby transmitting virus to the domestic poultry population, resulting in massive outbreaks.

Pathogenicity of NDV is determined by multiple traits as fusion protein cleavage site, linear epitopes of HN proteins, etc (Cho *et al*., 2008). Surprisingly, the deduced amino acid sequences of the F and HN envelope glycoproteins of the study isolates, showed a divergence ranging from 0.8% and 0.3–1.57% respectively, with the recent NDV strains isolated during April 2015 and June 2016 (Gowthaman *et al*., 2018). This finding highlights the frequency of mutations in the envelope glycoproteins resulting in the emergence of antigenic variants.

In India, chickens are commonly vaccinated against NDV infection by lentogenic strains such as LaSota, B1, F and mesogenic strains such as Komarov, R2B and Mukteshwar. The deduced amino acid sequences of full-length F and HN proteins of the study isolates showed a divergence ranging from 10–12.9% and 11.3–14.5% with the current vaccine strains. Reports from previous studies were in agreement with the present findings (Das and Kumar, 2017; Nath and Kumar, 2017; Gowthaman *et al*., 2018). Though the neutralizing epitopes in fusion protein are quite similar to that of the vaccine strains, several amino acid substitutions were noticed in the fusion peptide, hypervariable region, heptad repeat regions and transmembrane domains. In addition, amino acid substitutions were also identified in the antigenic sites of HN protein. Heptad repeat motifs in fusion and HN proteins interact with each other and are critical for fusion activity. Mutations in these domains have profound influence on virulence and pathogenicity of the isolates (Dortmans *et al*., 2009). Mutations within the antigenic site of HN protein may result in failure of antibody recognition capability. Hence, the chicken- and pelican-origin isolates may be considered as new immune-response escaping antigenic variants involved in ND outbreaks despite the flocks being vaccinated regularly. Mukteshwar strain is observed to have more similarity with the study isolates when compared to other vaccine strains. However, whole genome sequencing may supplement the available data to support that the Newcastle disease virus is evolving constantly.

The chicken- and pelican-origin NDV isolates were characterized for their ability to induce infection in three-week-old chickens and transmit to naïve chickens. Infection resulted in 100% mortality in both the infected and sentinel birds with clinical signs, gross lesions and histopathological changes typical of virulent ND. This finding clearly demonstrates that chicken-origin and non-chicken-origin NDV isolates could infect chickens in a similar manner. In agreement to the present findings, 100% mortality was recorded in chickens experimentally infected with peafowl-origin isolate (Desingu *et al*., 2017). In contrast, Ferreira *et al*. (2019) reported that the pigeon and cormorant lineage viruses required a high challenge dose to infect chickens and did not transmit efficiently to contact birds, even when a high infectious dose was used. It is proven that pathogenicity also depends on host adaptability, as a goose- origin isolate was found to be more pathogenic to geese than a chicken-origin isolate of the same genotype (Xu *et al*., 2017).

Spot-billed pelican, from which the isolate was obtained, was apparently healthy indicating that these birds may act as asymptomatic carriers of the virulent NDV, and spread the virus during their seasonal patterns of migration. Most poultry around the wild bird sanctuary were raised in a free-range style and might contract infections from wild birds through the contamination of water and feed. Several studies reported accidental spillover of virus from poultry outbreaks to other bird species (Seal *et al*., 1998; Vijayarani *et al*., 2010; Garcia *et al*., 2013; Dimitrov *et al*., 2016). It has been suggested that the NDV's that spillover from wild/feral birds or other species into poultry continue to evolve as they are rapidly passaged in chickens (Garcia *et al*., 2013). Hence, wildlife-poultry interface is shown to present a major risk factor to the poultry industry and highlights the need for enhanced biosecurity in commercial poultry operations.

Genetic and clinico-pathological characterization of the NDV strains isolated from apparently healthy spot-billed pelican (MIG-9) and backyard chicken flock (SKLM-1) confirmed the probable spillover of viruses between domestic and wild birds.

Conclusion

The present report is the first of its kind delineating simultaneous isolation of identical NDV strains of subgenotype XIII 2.2 from a spot-billed pelican and backyard chickens from India; furthermore, this study also provides additional evidence for the extended host range for NDV including migratory birds like spot-billed pelicans. Hence, future studies need to be directed towards evolving novel vaccination regimes together with regular surveillance of migratory birds, aquatic water fowl and the domestic poultry to understand the dynamics and evolution of new NDVs.

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Supplementary information is available in the online version of the paper.

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SUPPLEMENTARY INFORMATION

Simultaneous detection of velogenic Newcastle disease virus of genotype XIII 2.2 from spot-billed pelican and backyard chicken: implications to the viral maintenance and spread

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Table S1. Details of primers used in the study

<table>
<tr><th>S.No</th><th colspan="2">Primer name</th><th>5' to 3' sequence</th><th>Product size</th><th>Reference</th></tr>
<tr><td>1.</td><td colspan="2">FPCS-F</td><td>GCAGCTGCAGGGATTGTGGT</td><td rowspan="2">365 bp</td><td rowspan="2">Nantha Kumar et al., 2000</td></tr>
<tr><td>2.</td><td colspan="2">FPCS-R</td><td>TCTTTGAGCAGGAGGATGTTG</td></tr>
<tr><td rowspan="6">3.</td><td rowspan="6">Complete fusion gene (1662 bp)</td><td>F1-F</td><td>CACTAAGATAGAGAAGAG GCACACC</td><td rowspan="2">1058 bp</td><td rowspan="6">Gowthaman et al., 2018</td></tr>
<tr><td>F1-R</td><td>TTATACAGTCCAATTCTCGCGCC</td></tr>
<tr><td>F2-F</td><td>AAAGAGGCATGTGCAAAAGCCCC</td><td rowspan="2">929 bp</td></tr>
<tr><td>F2-R</td><td>GTGTAGTGAGTGCACCTTCAGTCT</td></tr>
<tr><td>F3-F</td><td>GGGAGCCTAAATAATATGCGCGCC</td><td rowspan="2">815 bp</td></tr>
<tr><td>F3-R</td><td>GCG CCA TGT ATT CTT TGC TTC</td></tr>
<tr><td rowspan="4">4.</td><td rowspan="4">Complete HN gene (1716 bp)</td><td>HN ext-F</td><td>AGAACG GTCAAAGGAGCCAC</td><td rowspan="4">1860 bp</td><td rowspan="4">Designed for this study</td></tr>
<tr><td>HN ext-R</td><td>GGTGCAACACCTTCCTTCCA</td></tr>
<tr><td>HN int-F</td><td>TCAATACTTGGCACTCGGTG</td></tr>
<tr><td>HN int- R</td><td>TTAGGTGGAACAGTCAGCAC</td></tr>
</table>

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Table S2. NDV reference sequences used in this study for phylogenetic analysis and evolutionary distance estimation

S.No	AAvV-1 isolate name	Genotype	GenBank ID
1.	AAvV-1_isolate/ I-2	I	AY935499.1
2.	AAvV-1_Strain/KOMAROV	II	KT445901.1
3.	AAvV-1_Strain/LASOTA	II	AF077761.1
4.	AAvV-1_Strain_B1	II	AF309418.1
5.	AAvV-1_Strain/R2B	II	JX316216.1
6.	AAvV-1 isolate D58 fusion protein (F) gene	II	EU330230.1
7.	AAvV-1 isolate D58 fusion protein (HN) gene	II	EU305607.1
8.	NDV turkey/USA/VGGA/89	II	AY289002.1
9.	NDV_ STRAIN _ F	II	KC987036.1
10.	AAvV-1_Strain_Mukteswar	III	EF201805.1
11.	AAvV-1_Isolate/JS/9/05/Go	III	FJ430160.1
12.	AAvV-1_Strain/ITALIEN	IV	EU293914.1
13.	NDV_STRAIN_ Beudette	IV	X04719.1
14.	AAvV-1_Isolate_2K3/Chennai/Tamil_Nadu/2000	IV	FJ986192.1
15.	NDV_strain_Ulster	IV	M24694.1
16.	NDV_strain_Texas_GB	IV	M33855.1
17.	NDV-2/chicken/Namakkal/Tamil Nadu,	IV	GU187941.1
18.	AAvV-1_Strain/ANHINGA/US_(Fl)/44083/93	V	AY562986.1
19.	APMV-1/chicken/Ca/2098/71,	V	JQ247691.1
20.	Newcastle disease virus isolate chicken/N. Ireland/Ulster/67, complete genome	V	AY562991.1
21.	AAvV-1_Isolate_ZhJ-3/97	VI	FJ766529.1
22.	NDV/ Japan/Ibaraki/85	VI	AB465606.1
23.	AAvV-1_Isolate_HN-7-06-Ch	VII	GQ245799.1
24.	NDV/ YZ-22-07-Os	VII	GQ245818.1
25.	AAvV-1_Strain_AF2240-I	VIII	JX012096.1
26.	AAvV-1_Strain/BLACKBIRD/CHINA/08	IX	KC934169.1
27.	NDV strain ZJ/1/86/Ch, complete genome	IX	FJ436303.1
28.	AAvV-1_Strain_Mallard/US(OH)/04-411/2004	X	GQ288377.1
29.	NDV virus strain northern pintail/US(OH)/87-486/1987	X	GQ288378.1
30.	AAvV-1_Strain/MG/1992	XI	HQ266603.1
31.	NDV strain MG_725_08, complete genome	XI	HQ266602.1
32.	AAvV-1_Strain_AAVV-1/Peacock/Peru/2011	XII	KR732614.1
33.	AAvV-1_Strain_CHICKEN/SWEDEN/97	XIII 1.1	GU585905.1
34.	AAvV-1 Ostrich/South Africa/45445-3/1995	XIII1.1	JN942034.1
35.	NDV_Isolate/COCKATOO/INDIA/7847/1982	XIII 1.1	JN942041.1
36.	NDV_Strain/CHICKEN/4132-20/BURUNDI/2008	XIII 1.1	FJ772494.1
37.	NDV_Isolate/BAREILLY/2013	XIII 1.1	KF727980.1
38.	NDV_Strain/BAREILLY/2011	XIII 1.1	HQ589257.1
39.	AAvV-1_Strain_CHICKEN/IRAN/EMM/1/2008	XIII 1.2	JQ267585.1
40.	AAvV-1_Chicken/Bareilly/01/10	XIII 1.2	KJ577585.1
41.	NDV_Strain/CHICKEN/IRAN/EMM/7/2011	XIII 1.2	JQ267579.1
42.	AAvV-1_Isolate/CHICKEN/CP/PAKISTAN/2010	XIII 2.1	JN682211.1
43.	AAvV-1_ isolate chicken/BYP/Pakistan/2010	XIII 2.1	JN 682210.1
44.	NDV strain chicken/Pakistan/NDV/UDL8/2011	XIII 2.1	JQ517285.1

Table S2. Continued

S.No	AAvV-1 isolate name	Genotype	GenBank ID
45.	NDV_Isolate/CHICKEN/CP/RAWALPINDI2/2010	XIII 2.1	JN682189.1
46.	AAvV-1 isolate 410/16A	XIII 2.2	MF422129.1
47.	AAvV-1 isolate 96-15	XIII 2.2	MF422125.1
48.	AAvV-1_Isolate/AAVV-116/GODHRA/03/2013	XIII 2.2	KM056344.1
49.	NDV isolate ndv52/Sarsa	XIII 2.2	KM056350.1
50.	NDV isolate D162 fusion protein (F) gene	XIII2.2	KX242342.1
51.	NDV strain Nagpur	XIII 2.2	KP089979.1
52.	NDV isolate NDV/Chicken/Nagpur/04/11	XIII 2.2	KX372708.1
53.	NDV isolate Pandu fusion protein (F) and hemagglutinin-neuraminidase (HN) genes	XIII 2.2	KT734766.1
54.	NDV_Strain/CHICKEN/RANCHI/01/2014	XIII 2.2	KR072665.1
55.	AAvV-1_Strain_Turkey/Nigeria/NIE09-2071/2009	XIV	HF969205.1
56.	AAvV-1 strain B/XJ/9/2009	XV	JF930146.1
57.	AAvV-1_Isolate_XJ-2/97	XV	AF458011.1
58.	NDV strain SD/5/04/Go fusion protein	XV	DQ682445.1
59.	AAvV-1 Strain/Chicken/Dominican_Republic/499- 31/2008	XVI	JX119193.1
60.	AAvV-1 strain chicken/DominicanRepublic/28138-4/1986	XVI	JX915242.1
61.	AAvV1_Strain_Chicken/Central_African_Republic/CAF09-015/2008	XVII	HF969181.1
62.	NDV strain chicken/Central African Republic/CAF09-016/2008	XVII	HF969182.1
63.	NDV strain chicken/Nigeria/NIE09-2087/2009	XVII	HF969155.1
64.	AAVV-1_Isolate_2007/Mali/ML038/07	XVIII	JF966389.1
65.	NDV strain-1532-14-Mauritania-2006 fusion protein (F)	XVIII	FJ772455.1
66.	AAvV-1 chicken/Nigeria/OOT/4/1/N69/914/2009	XVIII	MH392227.2
67.	AAvV-1 DCCO/USA/A00874288/650/2010	XIX	MK673141.1
68.	AAvV-1 isolate Cormorant/Florida/41105/2012	XIX	KC433530.1
69.	AAvV-1/crested ibis/China/Shaanxi10/2010	XX	KC853020.1
70.	AAvV-1 isolate pigeon/Pakistan/Lahore/21A/2015	XXI	KX236100.1
71.	AAvV-1 isolate Pigeon/AJK/AW-p54/2018	XXI	MH717070.1
72.	AAvV-1 isolate Pigeon/Pak/Lahore/AW-2/2015	XXI	KU862298.1