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RESEARCH ARTICLE Indian Journal of Animal Research

Cytopathic and Molecular Characterization of Emerging Avian Avulavirus-1 (AAvV-1) from Jammu

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10.18805/IJAR.B-5488

ABSTRACT

Background: Recent outbreaks of Newcastle disease (ND) were recorded in fowls and pigeons flocks from March 2021 to September 2022 in Jammu. Therefore, the aim of this study was to evaluate and characterize the ND virus (AAvV-1) isolates in this region.

Methods: Reverse Transcriptsase Polymerase chain reaction **(**RT-PCR) was used for the confirmation of NDV by targeting the partial Fusion protein gene. Further, sequencing and molecular characterization of partial F protein gene was done to study phylogenetic, genotypic and pathotypic characteristics of all the isolates. Cytopathic Effect (CPE) on Chicken Embryo Fibroblast (CEF) cells was studied for two representative virus isolates.

Result: Six AAvV-1 isolates three from pigeons and the other three from chickens of different geospatial areas were detected and sequenced. Nucleotide comparison showed five of six isolates had close homology (99.6-100%). Deducted amino acid sequence at the F-protein cleavage site showed a ^{112}R-R-Q-K-R*F^{117} velogenic motif for all isolates. Two fowl isolates and three pigeon isolates belonged to velogenic genotype II strain. One fowl isolate belonged to velogenic genotype VII strain (sub-genotype VII.1.1) with unique nucleotide residues. CPE on CEF were noticeable after 2nd infective passage, characterized by disruption of monolayer, cell rounding, vacuolization and detachment. The isolation of velogenic genotype II strains from both pigeons and fowls and the documentation of genotype VII.1.1 with presence of mutated nucleotides may indicate interspecies transmissibility and emergence of mutant strains.

Key words: Avian avulavirus-1, Chicken embryo fibroblast, Fowl, Fusion protein, Genotype, Newcastle disease, Pathotype, Pigeon.

INTRODUCTION

Newcastle disease (ND) is an infectious viral disease and virulent strains of the virus produce severe consequences, making the disease reportable to the Office International des Epizooties (OIE). The causative agent for ND is Avian Paramyxovirus serotype-1 (APMV-1), presently designated as Avian Avulavirus-1 (AAvV-1), belonging to the genus *avian avulavirus* (AAvV) and the family Paramyxoviridae (Alexander *et al*., 1984). Pigeon paramyxovirus-1 (PPMV-1) is a virulent variant of AAvV-1 responsible for infection in racing pigeons (Alexander *et al*., 1984). For convenience, the former designation of the viruses has been followed throughout.

Newcastle disease is endemic in India and different pathotypes of the virus have been isolated and characterized from various avian hosts (Akter *et al*., 2023). It has been reported from Jammu and Kashmir in both chickens and pigeons (Maqbool *et al*., 2016; Sangha, 2017; Mehmood *et al*., 2021; Chowdhary *et al*., 2020b). Although molecular characterization and genotype of prevalent strains have been documented (Maqbool *et al*., 2016; Chowdhary *et al*., 2020a), however, it was felt necessary to survey newer genotypes and pathotypes in circulation amongst normally resident birds.

Recently, outbreaks of Newcastle disease around Jammu region encompassing sixteen flocks of domesticated pigeons (*Columba livia domestica*) with 89.66% morbidity and 81.8% mortality and five flocks of backyard fowl (*Gallus*

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How to cite this article: Andrabi, S.A., Nashiruddullah, N., Brahma, B., Nehru, D.A., Ahmed, J.A., Sharma, V. and Roychoudhary, P. (2025). Cytopathic and Molecular Characterization of Emerging Avian Avulavirus-1 (AAvV-1) from Jammu. Indian Journal of Animal Research. 1-10. doi: 10.18805/IJAR.B-5488.

Submitted: 04-09-2024 **Accepted:** 31-01-2025 **Online:** 12-03-2025

gallus domesticus) exhibiting 100% morbidity and 91.5% mortality was recorded (Andrabi *et al*., 2023). Pigeons suspected of Newcastle disease showed predominant neurological signs and fowl flocks showed principal respiratory symptoms as described previously, with preliminary examination showing them to be velogenic based on Mean Death Time (MDT) estimation (Andrabi *et al*., 2023). The present study was carried out to characterize the virus strains isolated from the recent outbreak through cytopathic evaluation, gene sequencing, pathotyping and in-silico analysis.

MATERIALS AND METHODS

Clinical sample and preparation of viral antigen

The work has been carried out at Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu, Ranbir Singh Pura-181 102, Jammu and Kashmir, India. The materials for virus detection included oropharyngeal swabs from live birds and tissue homogenates from post-mortem samples (brain, lung, spleen, proventriculus) collected from suspected Newcastle disease (ND) outbreaks in sterile PBS (1:10 dilution) as per recommendations prescribed in OIE (2012). Samples were collected from March 2021 to September 2022. The samples (tissue suspensions and swabs) were clarified by centrifugation at 1,000× and supernatant was collected and filtered through 0.25 µm sterile syringe filter (Millipore Membrane Filter). All the selected samples were treated with mixture of penicillin and streptomycin (HyClone antibiotic, 100X) and incubated for one hour at 37°C in a Biological Oxygen Demand (BOD) incubator.

Virus isolation in chicken embryonated egg (CEE)

Nine to ten days old embryonated chicken eggs, from Govt. hatchery, Jammu were acquired and maintained in the egg incubator after candling and prepared for egg inoculation by allantoic route of inoculation (FAO, 2002). Allantoic fluid from infected eggs was also processed for virus detection by RT-PCR.

Virus isolation in chicken embryo fibroblast cell (CEF)

Viable 9-10 days old embryonated chicken eggs were washed with 70% alcohol and the embryo was retrieved and rinsed 3-4 times with PBS. Fibroblastic tissue was finely dissected and minced with scalpel and washed several times with PBS to remove any debris and blood vessels. The minced tissue was placed onto 35×10 mm tissue culture disk (Nunc, Roskilde, Denmark) and briefly heated to facilitate adherence to substrate. Complete Dulbecco2 s Modified Eagle2 s Medium.

(DMEM medium) was carefully added to the culture plates and maintained in a CO_2 incubator (Innova ® CO-170; Thermo Fisher Scientific Inc., PA, USA) at 37°C and 5 % CO_2 and appropriate humidity. Cell morphology and growth was monitored regularly. After 5-7 days at ~70% confluence, the cells were harvested using Trypsin-EDTA (0.25%). Further, the cells were sub cultured by seeding in 75 cm² culture flasks.

The flasks containing healthy CEF cells were infected with virulent NDV (genotype II and genotype VII) isolated from pigeon (NDV/P1/22/RSP-2) and poultry (NDV/F2/22/RSP-2) respectively and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 1 hour. After that, 25 ml fresh DMEM medium containing 2% heat inactivated FBS was added. One flask was mock infected with sterile PBS and used as a negative control. Cells were regularly checked for cytopathic effects in both the flasks.

[DMEM F-12 with $NaHCO_3$ (0.2%, w/v), HEPES (15 mM) and L-Glutamine (10 mM), 10 µg/ml ITS liquid media supplement (containing 1.0 mg/ml insulin, 0.55 mg/ml transferrin and 0.5 µg/ml sodium selenite), 1 µg/ml hydrocortisone Solution 10 ng/ml EGF and antibiotics (Penicillin-G-100 IU/ml, Streptomycin-5 µg/ml, Amphotericin-50 ng/ml) was used. The pH was adjusted to 7.4 before adding 10per cent foetal bovine serum (FBS). Complete medium was then filtered with 0.22 µm syringe filter].

RNA extraction and cDNA synthesis

For molecular detection of NDV, total RNA was extracted from oropharyngeal swabs and pooled tissue homogenates using TRIzol Reagent (Sigma-Aldrich, India). Complementary DNA was synthesized from total RNA using a commercial kit (Maxima H Minus Double Stranded cDNA synthesis kit; Thermo Fisher Scientific Inc., USA. #K1622).

Reverse transcriptase PCR

RT-PCR was carried out using NDV genome-specific primers for F gene *viz* Forward (F) primer: 5′GTCAATCATAGTC AAGTTGCTCCCGAATATGC3′ and Reverse (R) primer: 5′ ACTTCATGCACAGCTTCATTGGTTGCAGC3′ designed from available EST sequences at NCBI database with 327 bp length. Primer specificity was checked by nucleotide BLAST and UCSC BLAT tools. Detection by the primer pair was cross validated with standard vaccine virus and previously established primers (Yang *et al*., 1999).

A PCR buffer containing 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 U/µl Taq DNA polymerase and 10 pmoles of each primer was used for 25 µl PCR reaction. The PCR was conducted with initial denaturation (5 min at 94°C) followed by 32 cycles of denaturation (30 seconds at 94°C), primer annealing (45 seconds at 53°C), extension (1 min at 72°C) and a final extension (5 min at 72°C). Products were electrophoresed in 1.5% w/v agarose and visualized in a Gel Documentation System (BioDoc Analyze, Biometra, Germany).

Gene sequence and phenotypic analysis

The amplified products were gel purified to remove any non-specific DNA and primer-dimers employing PCR Clean-up Gel Extraction kit (Nucleo-pore, Genetix, #NP-36105). After proper labelling, the purified PCR products were sent for commercial sequencing facility at Biologia Research India

Pvt. Ltd, Mehruli Kishangarh, India-110070 and sequencing results were annotated and submitted to Genbank, NCBI for accession numbers.

Sequence comparison of the Newcastle disease virus F gene (partial regions) of the NDV genome, based on F gene cleavage site using open source BLAST program (National Center for Biotechnology Information, Bethesda MD, http://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequence comparison. Alignment of nucleotides performed by clustalW multiple alignment method.

The subsequent phylogenetic analysis and the percentage of nucleotide sequence similarity were determined by using NEIGHBOR-JOINING programme MEGA version 10.0 and the tropical accuracy of phylogenetic tree was estimated by 1000 bootstrap replicates (Tamura *et al*., 2011). For comparison, different sequences comprising all the notified genogroups, isolates from India, neighbouring as well as from other countries and vaccine strains were used to draw phylogenetic tree.

Molecular pathotyping

Deduced amino acid sequence pattern of the of the F gene cleavage site (111-117 amino acid position) was carried out (Aldous *et al*., 2003). Amino acid sequences were compared with other notified sequences including the vaccine strains by clustalW programme of MEGA 10.0.

RESULTS AND DISCUSSION

Reverse transcriptase PCR (RT-PCR) of Fusion protein gene

Confirmation of the virus was made by detection of NDV genome-specific primers designed for F gene, which yielded a product size of 326 bp from all processed samples as visualized on agarose gel (Fig 1). The positive samples were also cross validated using reported primers.

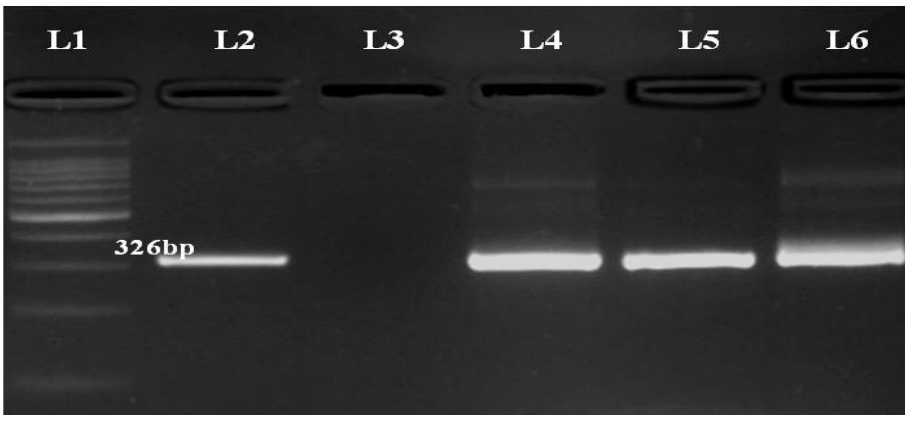
Cytopathic effect of CEF cells following NDV infection

Processed inoculum of fowl isolate (Genotype VII, NDV/F2/22/RSP-2) and pigeon isolate (Genotype II, NDV/P1/22/RSP-2) were inoculated on CEF cell monolayer after 5th passage of culture. CPE evident for both fowl and pigeon isolate were fairly similar (Table 1). In the first passage post-infection (ppi), infectivity with NDV was inapparent. Cytopathic effects were noticeable after 2nd passage of infection, characterized by disruption of monolayer, cell rounding, vacuolization and detachment (Fig 2). Changes were accelerated in the 3rd passage after virus inoculation.

Gene sequence analysis and comparison between NDV strains

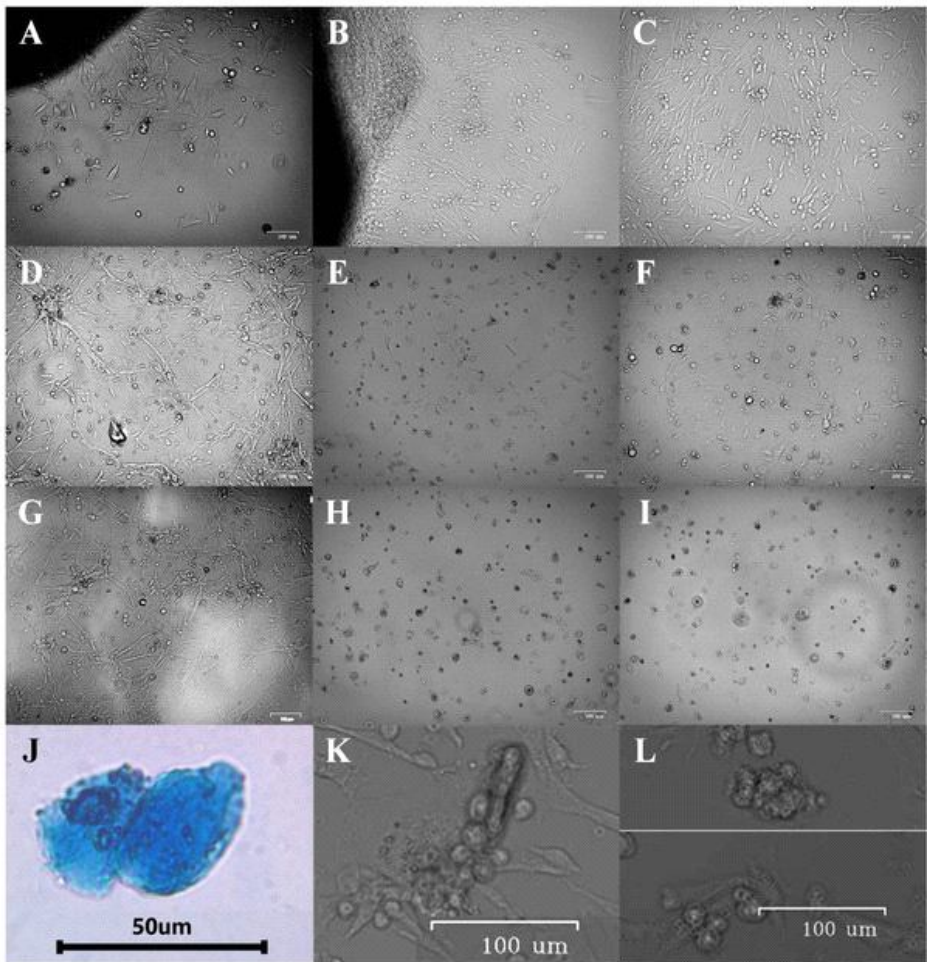
Three representative isolates each from fowls and pigeons belonging to the distinctive geo-spatial areas were selected for nucleotide sequencing and sequences were submitted in the open access, annotated public library- GenBank to allocate accession number for nucleotide and amino acid sequences (Table 2). Comparison of the partial *F* gene nucleotide sequences of pigeon and fowl isolates with reference strains, as shown in the (Fig 3) revealed nucleotide homology of 100% between NDV/P1/22/RSP-2 and NDV/P2/22/RSP-2 and 99.6% with NDV/P3/22/RSP-2. One of the fowl isolates NDV/F3/22/RSP-2 was 100% homologous to NDV/P1/22/RSP-2 and NDV/P2/22/RSP-2 and 99.6% homologous to NDV/P3/22/RSP-2. The previously isolated NDV from pigeon in Jammu (MH577764) region was 96.5% homologous to NDV/P1/22/RSP-2 and NDV/P2/22/RSP-2 and 99.8% homologous to NDV/P3/22/RSP-2. NDV/F1/22/RSP-2 shared 99.6% homology whereas NDV/F2/22/RSP-2 shared 69.6% homology with all the three pigeon isolates respectively.

Meanwhile, all the pigeon isolates along with the two fowl isolates clustered within genotype II, forming a separate



Lane (L1) 100 bp DNA ladder; (L2) Positive control (LaSota live vaccine virus); (L3) Negative control; (L4) Fowl tissue homogenate; (L5) Allantoic fluid inoculated with fowl isolate; (L6) Pigeon tissue homogenate; (L7) Allantoic fluid inoculated with pigeon isolate.

Fig 1: Reverse transcriptase PCR amplification of partial fusion protein gene encompassing the cleavage site with designed primers.



Uninfected cells: (A) Explant growth at 3 days; (B) Explants expansion at 5 days; (C) Third passage monolayer at 40% confluence.

CPE of cultured cells infected with NDV genotype-II (NDV/P1/22/RSP-2) of pigeon origin: (D) Disruption of monolayer at 24 hours post-infection (hpi); (E) Vacuolization, rounding and shrinkage, loss of cell attachment (hpi); Detachment of cells at 36 hpi; (F) Cell degeneration and detachment at 48 hpi.

CPE of culture cells infected with NDV genotype-VII of (NDV/F2/22/RSP-2) chicken origin: (G) Disruption of monolayer at 24 hours post-infection (hpi); (H) Vacuolization, rounding and shrinkage, loss of cell attachment at 36 hpi; (I) Cell detachment at 48 hpi. Higher magnification of infected cells: (J) Rounded fibroblast cells (Methylene blue stain); (K) Syncytia-like formation; (L) Composite frames showing rounding and vacuolization of cells.

Fig 2: Cytopathic effect of NDV in primary chicken fibroblast cells.

Table 1: Cytopathic effect (CPE) in chicken embryo fibroblast (CEF) infected with NDV.

Isolate	Cytopathic effect at 2nd passage post infection		
	24 hpi	36 hpi	48 hpi
Fowl isolate (Genotype VII)	Disruption of monolayer	Vacuolization, rounding and shrinkage, loss of cell attachment, Detachment of cells	Cell degeneration and detachment
Pigeon isolate (Genotype II)	Disruption of monolayer	Vacuolization, rounding and shrinkage, loss of cell attachment, Detachment of cells	Cell degeneration and detachment

clade. However, one of the fowl isolate NDV/F2/22/RSP-2 clustered in genotype VII that is a Group of highly velogenic NDV (Fig 4).

Molecular pathotyping

Based on deduced amino acid sequences between positions 110 to 118 for the Fusion gene cleavage site (at position 117, the N-terminus of F_1), the fowl and pigeon isolates were pathotyped accordingly (Fig 5). Both the fowl and pigeon isolates had a motif ^{110}G-G-R-R-Q-K-R*F-I^{118} which revealed that these isolates from fowls and pigeons were of velogenic pathotype.

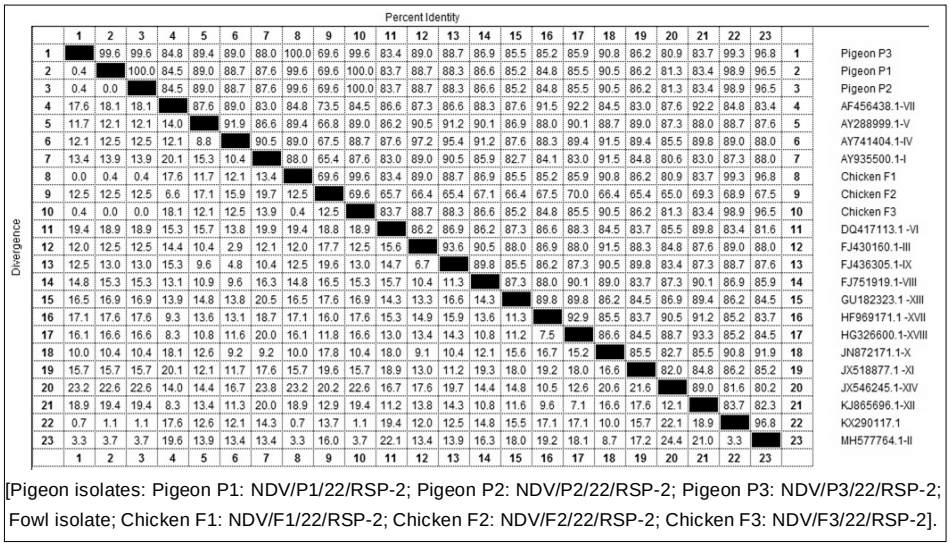
Comparison of the F gene sequences of the NDV-F2-17-RSP-1 with the reference strains revealed nucleotide homology varying between 88.2%-94.1% (Fig 6). NDV-F2-17-RSP-1(ON918571) sequence had the highest homology of 94.1% with NDV isolated from Iran (KX268351) and lowest homology with MF622047, the isolate from Southern Africa (88.2%). Phylogenetically, the fowl isolate NDV-F2-17-RSP-1 clustered under sub-genotype VII-I which has been also designated under clade VII.1.1 (Fig 7). Upon alignment with other genotype VII isolates, it was found to be unique at various nucleotide positions (Fig 8).

Jammu region is endemic for Newcastle disease and has been documented so far in backyard chicken and pigeons. In spite of the regular outbreaks, the actual prevalence of the disease in backyard fowl and pigeons is difficult to ascertain. The earlier pigeon outbreaks reported by Chowdhary *et al*. (2020a) were characterized as a lentogenic genotype-II PPMV-1, whereas the present findings in pigeons revealed genotype-II velogenic PPMV-1 strains.

The primers successfully detected NDV in the clinical samples from both fowl and pigeons and have been cross validated with standard vaccine virus and previously established primers (Yang *et al*., 1999). The present primer set could therefore be used for detection of both APMV-1 and PPMV-1.

Strains with low virulence do not have the ability to replicate in the absence of trypsin in the cell culture. However, both isolates were able to easily replicate in absence of trypsin, explaining their virulent pathotypes.

Sequencing and phylogeny revealed that one fowl isolate belonged to genotype VII which is a group of highly virulent NDV circulating across the globe and predominantly reported from the Asian countries (Shabbir *et al*., 2012).

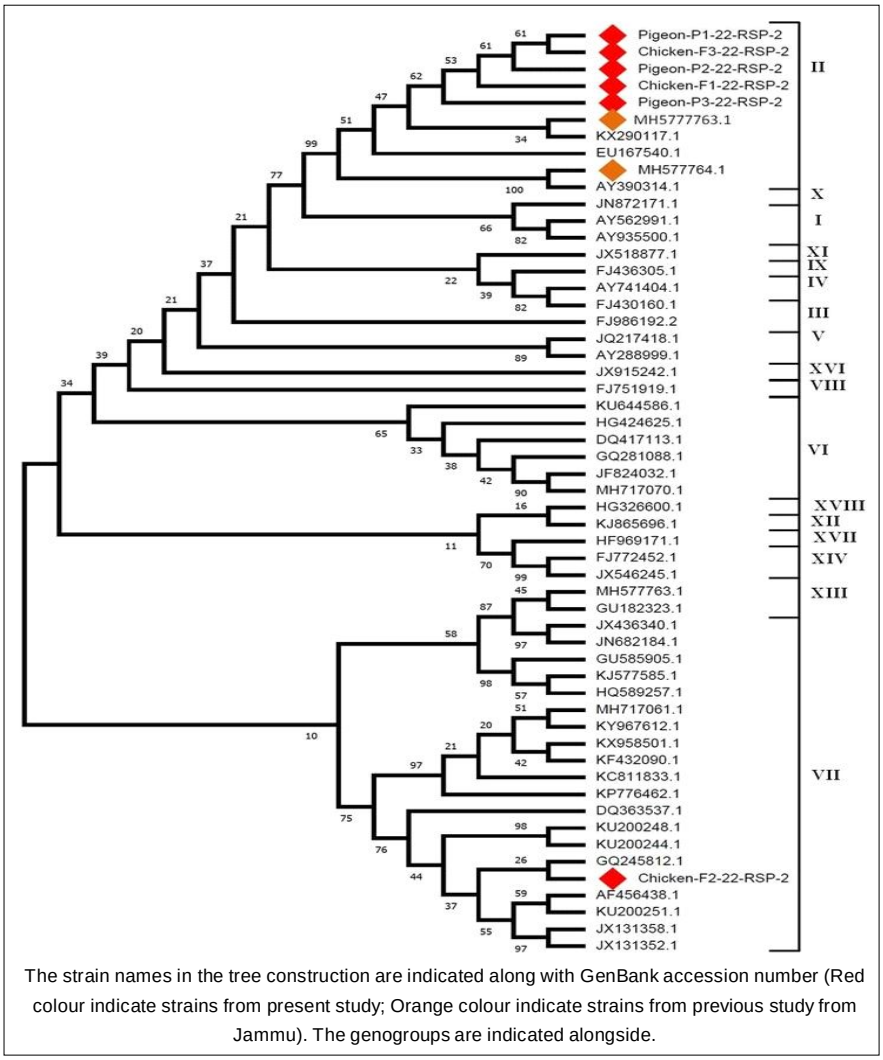


[Pigeon isolates: Pigeon P1: NDV/P1/22/RSP-2; Pigeon P2: NDV/P2/22/RSP-2; Pigeon P3: NDV/P3/22/RSP-2; Fowl isolate; Chicken F1: NDV/F1/22/RSP-2; Chicken F2: NDV/F2/22/RSP-2; Chicken F3: NDV/F3/22/RSP-2].

Fig 3: Alignment of nucleotides of fowl and pigeon isolates and their% identity/ divergence.

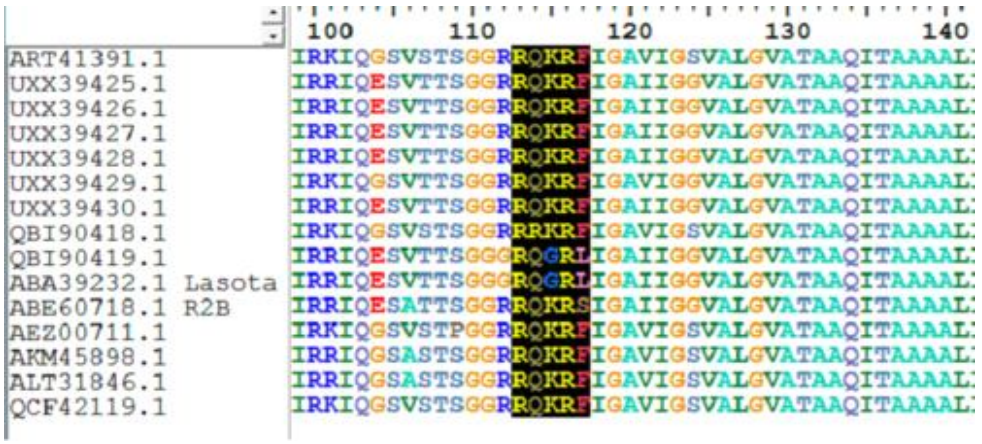
Table 2: Submission of nucleotide sequences in public library GenBank.

Isolate	Laboratory code	GenBank nucleotide accession number	GenBank protein accession number
Pigeon isolate	NDV/P1/22/RSP-2	ON918567	UXX39425.1
Pigeon isolate	NDV/P2/22/RSP-2	ON918568	UXX39426.1
Pigeon isolate	NDV/P3/22/RSP-2	ON918569	UXX39427.1
Fowl isolate	NDV/F1/22/RSP-2	ON918570	UXX39428.1
Fowl isolate	NDV/F2/22/RSP-2	ON918571	UXX39429.1
Fowl isolate	NDV/F3/22/RSP-2	ON918572	UXX39430.1



The strain names in the tree construction are indicated along with GenBank accession number (Red colour indicate strains from present study; Orange colour indicate strains from previous study from Jammu). The genogroups are indicated alongside.

Fig 4: Phylogenetic tree of representative members of *Avulavirus* using the Neighbour-joining method clustalW program of MEGA 10.0.



Pigeon isolates: UXX39425.1= NDV/P1/22/RSP-2; UXX39426.1= NDV/P2/22/RSP-2; UXX39427.1= NDV/P3/22/RSP-2.
Fowl isolates: UXX39428.1= NDV/F1/22/RSP-2; UXX39429.1= NDV/F2/22/RSP-2; UXX39430.1= NDV/F3/22/RSP-2.

Fig 5: Deduced amino acid motif at cleavage site of Fusion protein to determine virulence characteristic of fowl and pigeon isolates.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		96.6	96.6	95.4	96.6	96.6	93.7	95.8	94.9	93.7	94.9	89.9	91.6	92.0	1	AB853927.2
2	3.5		97.0	94.5	95.8	99.2	93.7	94.9	95.4	93.7	94.5	89.9	91.6	91.1	2	AY028995.1
3	3.5	3.0		94.5	95.8	97.0	93.7	94.9	94.1	93.7	94.1	89.9	91.6	91.6	3	DQ227246.1
4	4.9	5.7	5.8		97.0	95.4	92.4	93.7	96.2	92.4	95.4	89.5	90.3	92.4	4	EF579733.1
5	3.5	4.4	4.4	3.0		96.6	92.8	95.8	96.6	92.8	96.6	90.7	92.0	93.7	5	EF589133.1
6	3.5	0.8	3.0	4.8	3.5		93.7	95.8	95.8	93.7	94.9	90.7	92.4	92.0	6	GQ338309.1
7	6.7	6.7	6.7	8.1	7.7	6.7		93.7	92.0	100.0	92.0	89.0	90.3	90.3	7	HQ697254.1
8	4.4	5.3	5.3	6.7	4.4	4.4	6.7		93.2	93.7	94.1	92.4	92.4	92.0	8	JN986837.1
9	5.3	4.8	6.3	3.9	3.5	4.4	8.6	7.2		92.0	95.8	90.7	89.9	92.4	9	KC542905.1
10	6.7	6.7	6.7	8.1	7.7	6.7	0.0	6.7	8.6		92.0	89.0	90.3	90.3	10	KU862293.1
11	5.3	5.8	6.3	4.9	3.5	5.3	8.7	6.3	4.4	8.7		90.7	89.9	94.1	11	KX268351.1
12	11.2	11.1	11.2	11.6	10.1	10.1	12.1	8.1	10.1	12.1	10.1		87.3	89.5	12	KY747479.1
13	9.1	9.0	9.1	10.6	8.6	8.1	10.5	8.1	11.1	10.5	11.1	14.2		88.2	13	MF622047.1
14	8.6	9.5	9.1	8.1	6.7	8.6	10.6	8.6	8.1	10.6	6.2	11.6	13.1		14	ON918571.1 RSP-2
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Fig 6: Alignment of nucleotides of fowl NDV-F2-22-RSP-2 (ON918571.1) and its% identity/divergence with other genotype VII reference strains.

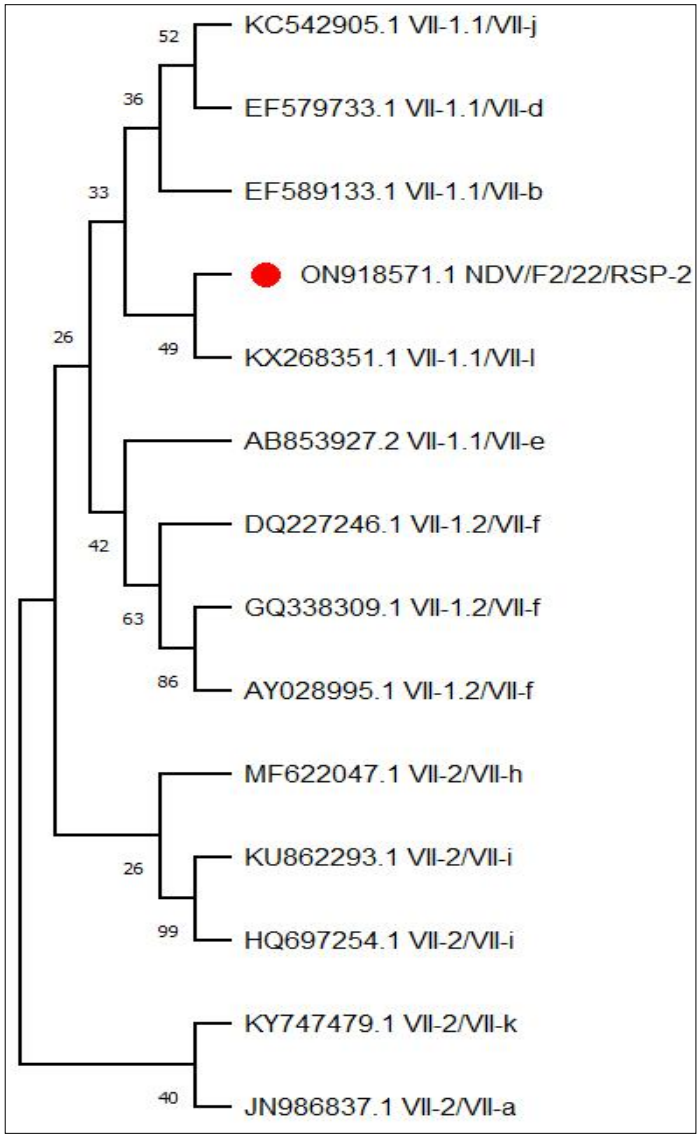


Fig 7: Rooted sub-tree for NDV-F2-17-RSP-1 (genotype VII) clustering under sub-genotype VII.1.1.

Genotype VII is one of the most variable genotypes of NDV which is continuously evolving thus forming the novel sub-genotypes. In addition, the isolate further clustered with the chicken isolate in a sub-genotype VII-I, proposed recently under clade 1.1 (VII.1.1) by Dimitrov *et al.* (2019). Previous studies have demonstrated the presence of virulent genotype XIII from chickens (Chowdhary *et al*., 2020b) in Jammu and genotype VII from chickens in Kashmir which were having close identity to Pakistan isolates, followed by China and Sweden strains (Maqbool, 2016). The emergence of novel virulent genotype VII.1.1 in the present study indicates that virus is continuously evolving in the field and leading to heavy mortalities among the poultry flocks. In India, prevalence of both genotype VII and XIII in chickens is well documented (Tirumurugaan *et al*., 2011; Jakhesara *et al*., 2014). Its noteworthy that genotype VII in general and sub-genotype VII.1.1 of NDV in particular is one of the emerging genotypes, with increasing isolation rates in poultry flocks in recent years (Xue *et al*., 2017). Thus, the present study highlights that genotyping of field isolates need to be carried out to undermine the epidemiological dynamics of evolving or newly emerging NDV strains, especially in context of virulent genotypes such as belonging to VII. The present VII.1.1 isolate was also found to have unique nucleotide residues at various positions when compared to other reference strains, thus revealing mutations of the wild type virus. These mutations underline possible evolution of newer variants with altered virulence and genotypic lineages.

In addition, identification of genotype II from the other two fowl and all three pigeon isolates indicate that it is the predominant genotype in circulation. It is noteworthy to mention that previous pigeon isolate was a lentogenic pathotype sharing 99.4% homology with LaSota vaccine virus and speculated as a spill-over of the vaccine strains into the environment (Chowdhary *et al*., 2020a). Surprisingly, the present genotype II isolates were all found to be velogenic with considerable homology to each other, while forming a separate clade from the previously isolate and LaSota. Genotype II is the only group which have both lentogenic and mesogenic pathotypes, apart from velogenic ones (Czegledi *et al*., 2002). Seemingly, a shift in pathotypes of genotype II in Jammu region since 2017 (NDV/P/17/RSP-1) corresponding proteolytic polybasic cleavage site (R-R-Q-K-R*F) have occurred. Such shifts have also been

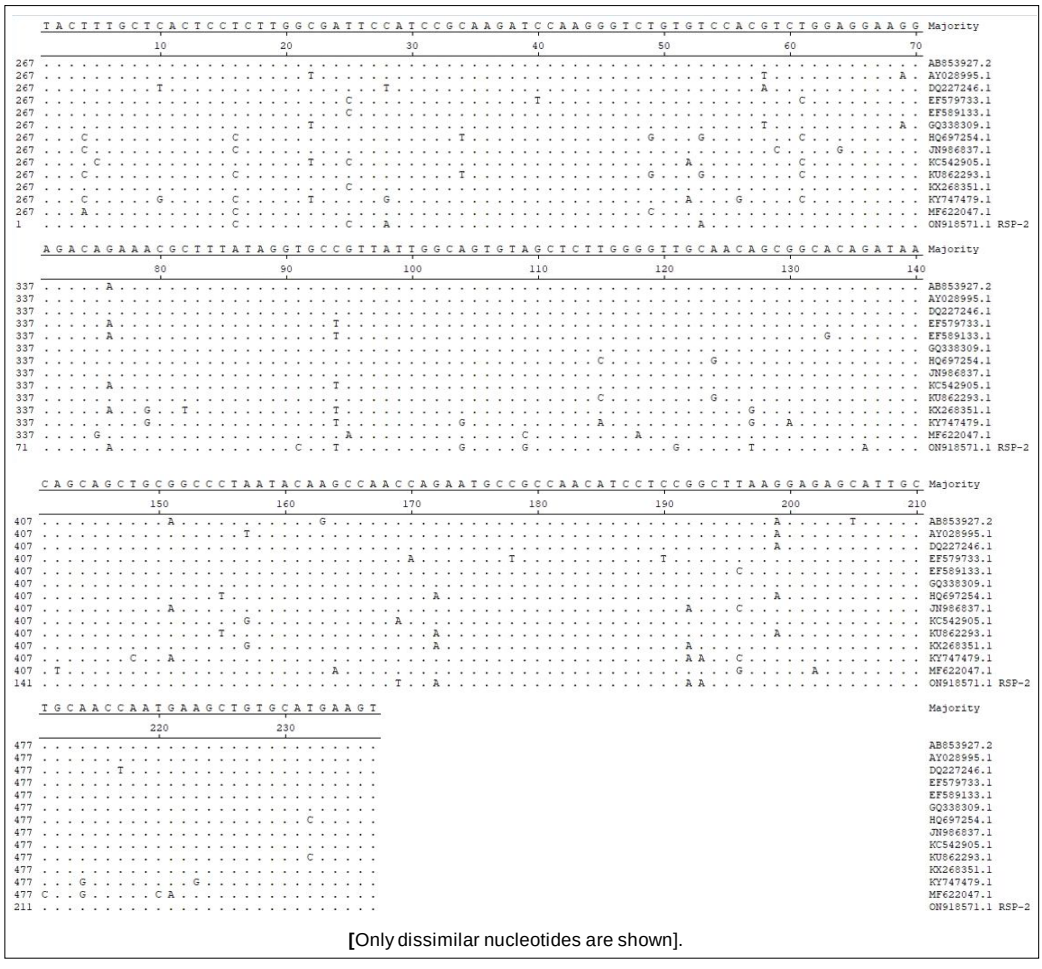


Fig 8: Deduced amino-acid sequence alignments of the F gene of fowl isolate NDV-F2-17-RSP-1 with other genotype VII reference strains.

documented elsewhere (Yu *et al*., 2001). Additionally, outbreaks with genotype II virus have recently been documented from other parts of the country as well (Bordoloi *et al*., 2021, Kochiganti *et al*., 2024).

Moreover, the phylogenetic closeness of two fowl and three pigeon NDV isolates (genotype II) under study hint towards an interspecies transmission potential of the virus.

In the light of the above speculations, it is a cause of great concern, if pigeons become potential reservoirs of NDV, they may disseminate avirulent viruses over time, which mutates to velogenic pathotypes evolving to recombinant strains.

Studies on NDV in Jammu and Kashmir indicated the concurrent prevalence of multiple genotypes with variable pathogenicity within the same state, *i.e*., velogenic and lentogenic genotype II in pigeons and virulent genotypes XIII and VII in fowls. This makes control strategies quiet challenging. Moreover, it has been reported that the strains which are of low virulence for pigeons or other avian species prove to be virulent for chickens as they have the property of selecting highly pathogenic derivatives from non-pathogenic precursors (Shengqing *et al*., 2002). In such an event of evolving recombinant strains, the efficacy of vaccine strains would remain questionable, unless trials are conducted to prove otherwise. Moreover, the concept of vaccinating the backyard fowls in the region is seldom practiced; while on the other hand, there has been an indiscriminate use of commercially available LaSota vaccines in pigeons, as admonished earlier (Chowdhary *et al*., 2020a).

CONCLUSION

It is concluded from the present study that apart from the circulating NDV strains in pigeons and fowls, there is a possibility of exotic strains within other wild free-flying birds in the study area (Gharana wetlands) and surrounding villages of the international border with Pakistan. The conditions could be just right for introduction and dissemination of new virus strains especially by free flying pigeons that cross the border. Speculations of virus cross-species and cross-border disease transmissibility may lead to continued virus evolution and persistent endemicity of the disease with serious consequences.

ACKNOWLEDGEMENT

Authors acknowledge the facilities and part funding under RCM project by Sher-e-Kashmir University of Agricultural Science and Technology, Jammu.

Conflict of interest

The authors declare no conflict of interests.

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