RESEARCH ARTICLE Indian Journal of Animal Research, Volume 57 Issue 5: 558-564 (May 2023)

Molecular Characterization of Velogenic Avian Avulavirus Type 1 Isolated from Apparently Healthy Emu Birds: Implications to Viral Maintenance and Spread

B. Deepthi[1], D. Ratnamma[1], R.N. Ramani Pushpa[1], Shrikrishna Isloor[1], B.M. Veeregowda[1], M.L. Satyanarayana[1]

10.18805/IJAR.B-4350

ABSTRACT

Background: Newcastle disease caused by Avian avulavirus type 1 (AAvV-1) is one of the dreadful diseases affecting poultry and other avian species. Wild birds and several domestic birds are recognized as reservoirs of AAvV-1 and probably contribute to the epidemiology of ND in the domesticated poultry. Hence, efforts have been made to understand the virulence and genetic nature of AAvV-1 isolates obtained from apparently healthy Emu birds.

Methods: This study details characterization of a velogenic Emu/5 AAvV-1 isolate obtained from an asymptomatic emu flock. Full- length fusion gene was amplified and subsequent phylogenetic analysis was performed. Experimental inoculation of 3-week old chicken with the isolate resulted in virulent ND. Expression of cytokine mRNA levels in spleen of infected chicken at different time points correlated well with the clinical picture, gross and histopathological lesions.

Result: To our knowledge this is the first evidence for the role of apparently healthy emu bird acting as a reservoir of velogenic AAvV-1 of subgenotype XIII 2.2 which proved to be highly virulent to chicken. This study further highlights the role of reservoir birds in AAvV-1 transmission and the need for adopting most realistic strategies in counteracting the disease.

Key words: *Avian avula virus-1*, Chicken, Cytokine expression, Emu, Pathogenicity, Transmission.

INTRODUCTION

Newcastle disease (ND) though identified nearly a century ago, still continues to be a major challenge to the poultry producers. Though several vaccination strategies are being adopted at the field level to control ND, outbreaks appear regularly either due to involvement of a new genotype, asymptomatic birds acting as reservoirs of virulent *Avian Avula virus-1* (AAvV-1) or due to inadequate vaccination. AAvV-1 belongs to genus *Avian orthoavulavirus 1* (AOAV-1) within the subfamily *Avulavirinae* of the family *Paramyxoviridae* (ICTV, 2019). AAvV-1 causes infections in a wide range of domestic and wild birds worldwide. As many as 241 species from 27 orders of the avian family are reported to be susceptible to ND (Kaleta and Baldauf, 1988). Fusion protein cleavage site (FPCS) is one of the most important virulence factors that distinguishes an isolate to be lentogenic or velogenic (OIE, 2012). Further, based on the full - length fusion gene sequences, AAvV-1 has been classified into two major classes, I and II. All the avirulent isolates were classified under Class I, which includes a single genotype with three subgenotypes. Class II contains most of the virulent viruses circulating worldwide, classified into 21 genotypes with multiple subgenotypes (Dimitrov *et al*., 2019) and this diversity continues to increase as the surveillance studies improves.

Presence of virulent viruses in healthy birds and their constant evolution over time suggests existence of a high environmental over load with continuous replication of AAvV-1 strains in endemic countries (Wajid *et al.,* 2017). Indian poultry farming systems presents a unique opportunity to study viral maintenance and spread. India has a wide variety of poultry species; however limited information is available regarding the potential of these avian species as asymptomatic reservoirs of AAvV-1. To understand the role of emu birds in the dissemination of potential pathogens, a surveillance study was undertaken in organized emu flocks of Andhra Pradesh and Telangana, India. Here we report isolation of velogenic AAvV-1 from apparently healthy emu birds which was proven to be highly pathogenic to chicken by infection studies.

[1]Department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram-521 102, Andhra Pradesh, India.

Corresponding Author: B. Deepthi, Department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram-521 102, Andhra Pradesh, India. Email: drdeepthib@gmail.com

How to cite this article: Deepthi, B., Ratnamma, D., Pushpa, R.N.R., Isloor, S., Veeregowda, B.M. and Satyanarayana, M.L. (2023). Molecular Characterization of Velogenic Avian Avulavirus Type 1 Isolated from Apparently Healthy Emu Birds: Implications to Viral Maintenance and Spread. Indian Journal of Animal Research. 57(5): 558-564. doi: 10.18805/IJAR.B-4350.

Submitted: 29-10-2020 **Accepted:** 08-01-2021 **Online:** 09-03-2021

MATERIALS AND METHODS

Ethical statement

Animal experimental protocols performed in the study are approved by Institutional Animal Ethics Committee of NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India (Approval number: 5/IAEC/NTRCVSC/2018).

Virus isolation and confirmation

During the years 2017 to 2019, both oropharyngeal and cloacal swabs (*n*=175) were collected from apparently healthy emu flocks of Andhra Pradesh and Telangana states. Swabs were collected in sterile containers with sterile phosphate buffered saline (PBS) pH 7.0 -7.4 containing antibiotics (100 units of penicillin G, 100 µg of streptomycin and 0.25 µg of amphotericin B/ ml), transported on ice to the lab of Department of Veterinary Microbiology, NTR College of Veterinary Science and stored at -80°C until processing. The samples were processed following standard protocols of virus isolation as specified in Terrestrial manual of OIE (OIE, 2012). In brief, the supernatant fluids of oropharyngeal and cloacal swabs were centrifuged at 5000 g for 10 minutes at 4°C and filtered. The filtrates were added with antibiotic- antimycotic solution and used for inoculation of 9-11 day old chicken embryos through allantoic cavities as previously described (OIE, 2012). The presence of AAvV-1 in allantoic fluid was confirmed by hemagglutination inhibition (HI) assay using polyclonal serum raised against LaSota and RT- PCR targeting fusion gene encompassing FPCS (OIE, 2012).

RNA extraction, cDNA synthesis, PCR and fusion gene sequencing

Viral RNA was extracted from the allantoic fluid using TRIzol LS reagent (Genei, Bangalore). iScript ™ cDNA synthesis kit (Biorad, USA) was used for synthesis of first strand cDNA using random hexamers. Presence of virus was confirmed by RT-PCR using primers targeting FPCS of fusion gene (Nantha Kumar *et al*., 2000). Complete fusion gene was amplified using consensus primers (Gowthaman *et al.,* 2018) and used for phylogenetic analysis and genotype determination. Services of commercial sequencing firm (Barcode Biosciences, India) were utilized for obtaining the sequence of complete fusion gene.

Pathogenicity indices and infection of CEF

The pathogenicity indices, including the mean death time (MDT) and the intra-cerebral pathogenicity index (ICPI) were determined as described previously (OIE, 2012). Virus infection of CEF cells was performed according to the methods described previously (Freshney, 2015).

Phylogenetic analysis

Homology searches were conducted using the NCBI program BLAST and AAvV-1 reference serotype sequences were retrieved from the GenBank data base. Multiple alignments of the sequences were performed using Clustal W in MEGA version X (Kumar *et al.,* 2018). Following multiple alignment, maximum likelihood values were determined to calculate percent identity and diversity, to generate phylogenetic trees and to determine the genotype of the isolate under study. The significance of all deduced phylogenetic trees were verified by bootstrap analysis of 1000 replicates. The maximum likelihood method based on the General Time Reversible (GTR) model with a discrete gamma distribution (five categories [+G]) was utilised for tree construction. All positions containing gaps and missing data were eliminated. There were a total of 1662 positions in the final dataset. The tree with the highest log likelihood (-15133.69) is shown. The evolutionary relationship between the Emu/5 isolate and 41 representative isolates of 21 genotypes of class II AAvV-1 were estimated by calculating the number of base differences per site from averaging over all sequence pairs between groups using MEGAX (Kumar *et al*., 2018). Details of the accession numbers used for phylogenetic analysis were mentioned in the supplementary material (S1).

Virus infection of chicken

The AAvV-1 isolate was further characterized to understand the early host immune response induced by the virus in chicken and to assess the risk of transmission to naïve chicken. A total number of thirty-one, 3-week-old broiler chicks were split into three groups *viz*., uninfected control group (*n*=14), Emu/5 inoculated group (*n*=14) and sentinel group (*n*=3). Control group was inoculated with PBS, while the experimental group was inoculated with a single dose of 100 µl allantoic fluid (HA titre of 1024 per 50 µl) through subcutaneous route as described in (OIE, 2012). Control group was placed in separate facility. Sentinel group was placed in a cage immediately next to the infected group to assess viral transmission from infected to un- infected birds. Infected birds were observed regularly for clinical signs and mortality. Three birds from each group were euthanized at 1, 3 and 5 day post infection (dpi) and spleen was collected in RNA later® (Sigma), for evaluating cytokine responses. Relative changes in RNA expression of pro-inflammatory cytokine genes (TNF-α, IL-1β and IL-6), chemokine (IL-8) and apoptotic gene (Cas-8) and IL-10, an anti-inflammatory cytokine and β- actin for infected and mock- infected controls were measured as changes in SYBR green fluorescence as described previously (Rajasekaran *et al.,* 2018). The remaining birds were monitored twice daily for clinical signs/ mortality upto 7 days. Necropsies were performed on the dead birds to examine for gross lesions. Tissues *viz.,* spleen, intestines, trachea, lung, kidney, liver and heart were collected in 10% neutral buffered formalin for histopathological analysis by routine paraffin embedding technique and microtomy. Four-micron thick sections were stained by hematoxylin and eosin method (Culling, 1968).

RESULTS AND DISCUSSION

In this study, we obtained AAvV-1 isolate (Emu/5, Genbank: MT178234) from an apparently healthy emu flock. Previous reports confirmed isolation of vNDV from an infected emu

S1: Reference AAvV-1 strains retrieved from GenBank along with accession numbers.

AAvV-1 isolate name	Genotype	GenBank ID
AAvV-1_Isolate/ I-2	I	AY935499.1
AAvV-1_Strain/KOMAROV	II	KT445901.1
AAvV-1_Strain/LASOTA	II	AF077761.1
AAvV-1_Strain_B1	II	AF309418.1
AAvV-1_Strain/R2B	II	JX316216.1
AAvV-1_Turkey/USA/VGGA/89	II	AY289002.1
AAvV-1_Strain_Mukteswar	III	EF201805.1
AAvV-1_Isolate/JS/9/05/Go	III	FJ430160.1
AAvV-1_Strain/ITALIEN	IV	EU293914.1
AAvV-1_Isolate_2K3/Chennai/Tamil_Nadu/2000	IV	FJ986192.1
AAvV-1_Chicken/Namakkal/Tamil Nadu, complete genome	IV	GU187941.1
AAvV-1_Strain/ANHINGA/US_(FI)/44083/93	V	AY562986.1
AAvV-1_Chicken/N. Ireland/Ulster/67, complete genome	V	AY562991.1
AAvV-1_1/chicken/Ca/2098/71, complete genome	V	JQ247691.1
AAvV-1_Isolate_ZhJ-3/97	VI	FJ766529.1
AAvV-1_Japan/Ibaraki/85	VI	AB465606.1
AAvV-1_Isolate_HN-7-06-Ch	VII	GQ245799.1
AAvV-1_YZ-22-07-Os	VII	GQ245818
AAvV-1_Strain_AF2240-I	VIII	JX012096.1
AAvV-1_Strain/BLACKBIRD/CHINA/08	IX	KC934169.1
AAvV-1_ZJ/1/86/Ch	IX	FJ436303.1
AAvV-1_Strain_Mallard/US(OH)/04-411/2004	X	GQ288377.1
AAvV-1_Northern pintail/US(OH)/87-486/1987	X	GQ288378.1
AAvV-1_Strain/MG/1992	XI	HQ266603.1
AAvV-1_MG_725_08, complete genome	XI	HQ266602.1
AAvV-1_Strain_AAVV-1/Peacock/Peru/2011	XII	KR732614.1
AAvV-1_Ostrich/South Africa/45445-3/1995	XIII 1.1	JN942034.1
AAvV-1_Strain_CHICKEN/IRAN/EMM/1/2008	XIII 1.2	JQ267585.1
AAvV-1_Chicken/Bareilly/01/10	XIII 1.2	KJ577585.1
AAvV-1_Isolate/CHICKEN/CP/PAKISTAN/2010	XIII 2.1	JN682211.1
AAvV-1_Isolate 410/16A	XIII 2.2	MF422129.1
AAvV-1_Isolate 96-15	XIII 2.2	MF422125.1
AAvV-1_Strain_Turkey/Nigeria/NIE09-2071/2009	XIV	HF969205.1
AAvV-1_Strain chicken/Nigeria/NIE09-2087/2009	XIV	HF969155.1
AAvV-1_Strain SD/5/04/Go fusion protein (F)	XV	DQ682445.1
AAvV-1_Chicken/DominicanRepublic/28138-4/1986	XVI	JX915242.1
AAvV1_Strain_Chicken/Central_African_Republic/CAF09-015/2008	XVII	HF969181.1
AAvV-1_Strain chicken/Central African Republic/CAF09-016/2008	XVII	HF969182.1
AAvV-1_-1532-14-Mauritania-2006	XVIII	FJ772455.1
AAvV-1_Isolate Cormorant/Florida/41105/2012	XIX	KC433530.1
AAvV-1_Pigeon/Pak/Lahore/AW-2/2015	XXI	KU862298.1

flock raised in a poultry dense area (Gowthaman *et al.,* 2016). Shinde *et al*. (2012) demonstrated that 15.3% of emus are seropositive in some regions of India. In general, emus are housed in open pens in Indian field conditions providing opportunity for interaction with other wild and feral birds.

The virus could be isolated during second passage in embryonated chicken eggs. Harvested embryos showed typical lesions such as sub-occipital haemorrhages and the allantoic fluid had a haemagglutination titre of 256-512 per 50 µl. HI titre with polyclonal chicken antiserum to LaSota was 1:128 thus confirming the isolate as AAvV-1. The AAvV-1 infective dose for a chicken is reported to range from $10^{3.0}$ (King, 1996) to $10^{4.0}$ EID_{50} (Alexander *et al*., 2006). Hence, it can be assumed that virus load harboured by the asymptomatic birds might be very low thus necessitating repeated passages in embryonated chicken eggs for isolation of the virus.

In addition, Mean death time score in 9-11 day old embryonated chicken eggs was 48 h and ICPI score in day-old chicks was 1.82, suggesting that the isolate was highly

virulent to chicken. The isolate was further characterized by propagating in chicken embryo fibroblasts (CEF) cultures. Characteristic cytopathic changes were observed in the first passage which included cellular granulation, syncytia formation, vacoulation and cytoplasmic process formation. Amplification and sequence analysis of the fusion gene revealed ^{112}R-R-R-K-R^{116}-F motif at the fusion protein cleavage site, typically found in velogenic AAvV-1. Full-length fusion gene was found to have a cds of 1662 nucleotides coding for 553 amino acids. Phylogenetic analysis based on the full- length fusion gene classified the virus as belonging to subgenotype XIII 2.2 (Fig 1). The Emu/5 isolate clustered closely with Avian avulavirus 1 isolate 410/16A (GenBank ID MF422129.1) and Avian avulavirus 1 isolate 96-15 (GenBank ID MF422125.1) isolated from Andhra Pradesh. The nucleotide and deduced amino acid sequence of fusion gene of Emu/5 isolate was aligned with representative sequences of all 21 genotypes and with the current vaccine strains and used in determination of pairwise comparisons and homologies. The percentage of nucleotide and deduced amino acid sequence identity of full- length F gene of the isolate with subgenotype XIII 2.2 isolates was 98.56% and 94.76%, while the nucleotide and deduced amino acid sequence identity with currently used vaccine strains was only 81.2% and 87.3% respectively. During 2002-2003, Kinde *et al.* (2005) reported isolation of exotic newcastle disease (END) virus from emus. Similarly, Gowthaman *et al.* (2016) reported isolation of velogenic AAvV-1 of subgenotype XIII 2.2 from an infected emu flock with a similar motif at FPCS. ND outbreaks in commercial chicken flocks caused by subgenotype XIII 2.2 viruses were also reported in different parts of India (Jhakesara *et al.,* 2016; Gowthaman *et al*., 2018).

The six neutralizing epitopes critical for structure and function of fusion protein (D72, E74, A75, K78, A79, L343) were found to be unaltered in the Emu/5 isolate when compared with the LaSota vaccine strain. Six potential N-glycosylation sites (Asn-X-Ser/Thr or N-X-S/T, where X present 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}and ^{541}NNT^{543} were also found to be conserved. of the eight trans membrane domains of AAvV-1 isolates located at positions 14-27, 15-25, 118-131, 120-128, 266-269, 429-432, 499-

Table 1: Amino acid differences in the heptad repeat regions of the fusion protein of LaSota and Emu/5.

HRa	HRb	HRc	HRd
K145N	G271A	N479D	D82E
	N272Y	E482A	R101K
	L282I	R486S	
	T288N	K487R	
	S291T	K494R	
	N294S		

*Amino acid residue substitutions at the indicated positions when LaSota vaccine strain is compared to Emu/5.

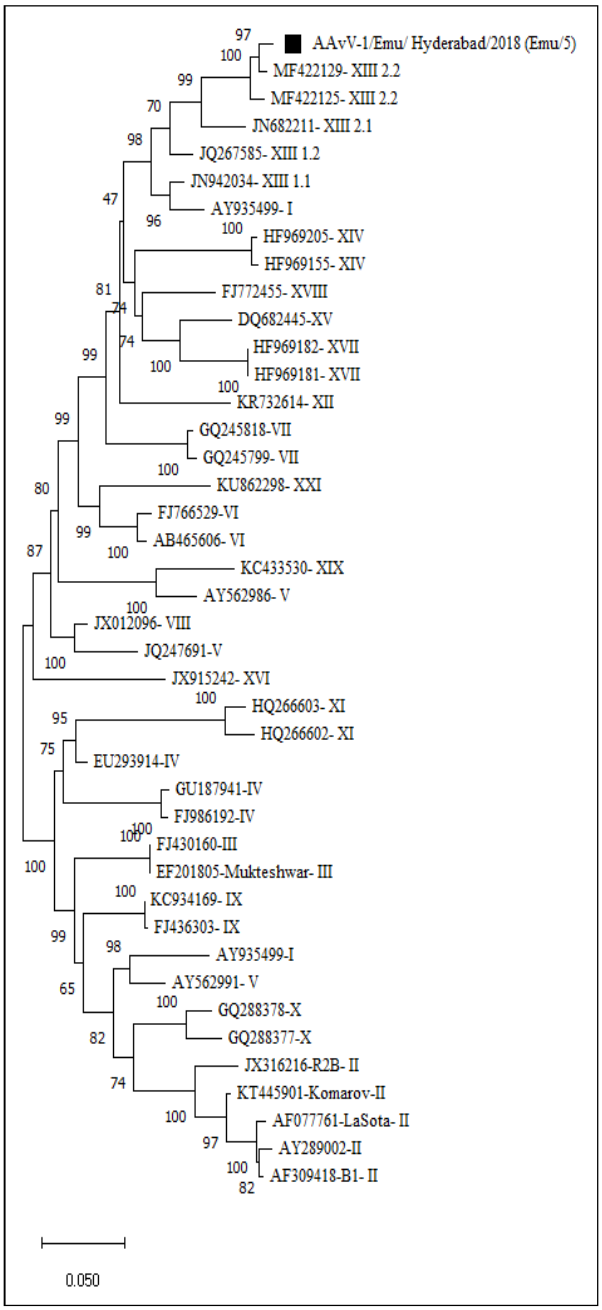

Fig 1: Phylogenetic analysis based on complete fusion gene: The evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-15133.69) is shown. The percentage of trees in which the associated taxa clustered together is shown 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 Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 42 nucleotide sequences. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

525 and 501-523 in the F protein, one substitution was observed at M14R. Further, several amino acid substitutions were observed in the heptad repeat regions (HRa, HRb, HRc and HRd) and hypervariable region of the fusion protein (Table 1 and 2). Heptad repeats have been shown to be critical for fusion and mutations in these regions decrease the fusion activity (Mc Ginnes *et al*., 2001). Significance of the mutations observed in the fusion gene and influence on pathogenicity needs further investigations. It is worth to note that an amino acid substitution (G271A) in HRb region was unique to Emu/5 isolate and not found in other subgenotype XIII 2.2 viruses.

Experimental inoculation of three- week old chicks with the Emu/5 isolate resulted in virulent ND with 100% mortality in the infected and sentinel groups. Infected birds were active and apparently healthy on 1dpi. Birds started exhibiting mild clinical signs such as lack of alertness and depression by 2 dpi, ruffled feathers, marked depression and drooping eyelids by 3 dpi. One bird was found dead on 4 dpi. By 5 dpi, marked clinical signs such as labored breathing, lateral recumbency and ataxia were seen in all birds. Gross lesions in tissues until 3 dpi, included diffuse mild haemorrhages throughout the carcass. Necropsy of the bird dead on 4 dpi, showed haemorrhages in the proventriculus, necrotic lesions in the intestines, subcutaneous edema and tracheal haemorrhages. Similar lesions were more pronounced in birds which succumbed later (Fig 2).

Tissues collected on 1dpi such as spleen, intestines, lungs, kidneys, trachea and heart did not show any pathological lesions. Mild haemorrhages were observed in tissues collected on 3 dpi. Tissues collected on 5 dpi revealed severe necrosis and lymphocytolysis of spleen, denudation of tracheal epithelium, vacoulation, mononuclear cell infiltration in trachea, hyperplasia and hypertrophy of bronchiolar epithelium and severe submucosal infiltration in lungs, severe necrosis and fusion of villi in the intestines, severe tubular necrosis in kidneys, pericarditis with infiltration of fibrin, fibroblasts and mononuclear cells in the heart (Fig 3).

Relative expression of cytokine genes in response to Emu/5 isolate are represented graphically (Fig 4). The expression levels of pro inflammatory cytokines, IL-1β and IL-6 induced were upregulated at 1 dpi and further upregulation was observed at 3 dpi by several folds and reduced by 5 dpi. Step-wise upregulation was observed in the expression of TNF-α from 1 to 5 dpi. The expression of Cas-8 is markedly upregulated and reached its peak by 5 dpi. The expression of anti- inflammatory cytokine, IL-10 gene was not detected during 1 and 3 dpi, but reached its peak and became significantly higher on 5 dpi. Gradual upregulation of IL-8 expression was observed from 1 dpi to 3 dpi, later down regulated by 5 dpi (0.57-fold). Pro inflammatory cytokines and chemokines were found to be upregulated during acute infection and responsible for tissue damage caused due to necrotic lesions in intestines and cytolytic changes in spleen. Il-10, an anti-inflammatory cytokine was upregulated during the later part of infection that has a role in dampening the inflammation and tissue

Table 2: Amino acid differences in the hyper variable region of the fusion proteins of Emu/5 isolate incomparison with vaccine strains.

Strains	Hypervariable region																	
	3	4	10	11	13	14	16	17	20	22	25	26	27	28	29	30	31	69
LaSota	S	R	P	A	M	M	T	I	A	V	C	I	C	P	A	N	I	L
R_2B	P	-	-	T	-	-	-	V	-	-	-	-	-	-	-	-	-	-
B_1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Komarov	P	-	-	T	-	-	-	V	-	-	-	-	-	-	-	-	-	-
Mukteshwar	P	-	-	V	L	-	-	-	T	A	Y	V	R	L	T	-	L	M
Emu/5	-	K	-	I	L	R	I	T	M	I	-	-	-	L	T	-	S	-

*Amino acids that match the consensus (LaSota strain) exactly are denoted by '-'.

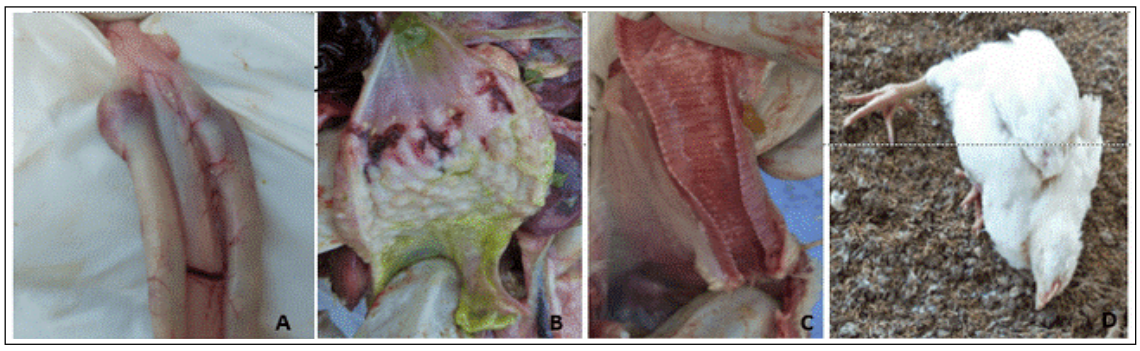

Fig 2: Clinical signs and gross lesions observed after infection of 3- week old chicks with Emu/5 isolate: A. Caecal tonsil necrosis with typical button ulcers; B. Hemorrhages at the tips of proventricular glands; C. Tracheal hemorrhages; D. Moribund chick with unilateral leg paralysis.

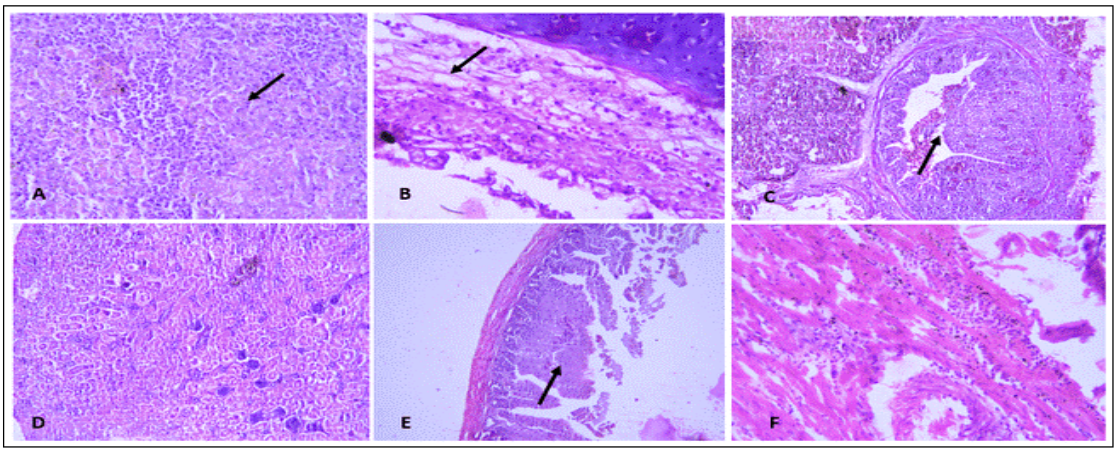

Fig 3: Histopathological lesions in H&E stained tissue sections of experimentally infected chicks. A. Spleen showing lymphoid depletion and diffuse necrosis (40X). B. Trachea showing vacoulation and denudation of epithelium (40X). C. Lung showing hypertrophy of bronchiolar epithelium (10 X). D. Kidney showing severe tubular necrosis (4X). E. Intestine showing severe necrosis and fusion of villi (10X). F. Heart showing pericarditis with infiltration of fibrin and fibroblasts (40X).

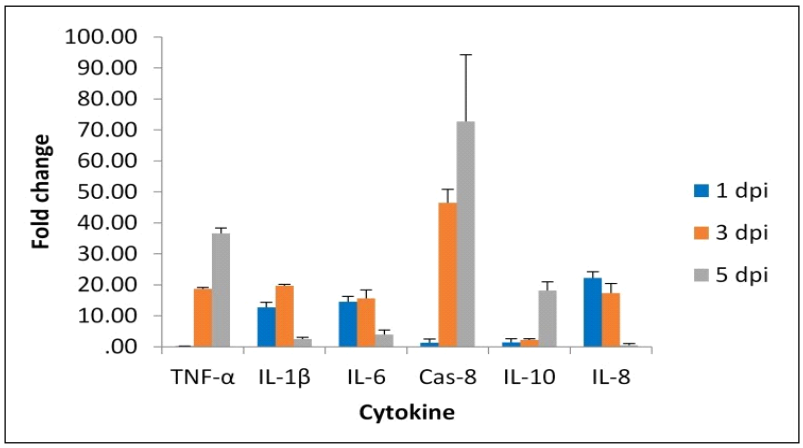

Fig 4: Expression of cytokine mRNA levels in spleen of infected chicken measured at 1, 3 and 5 dpi.

repair. Maximum upregulation of proinflammatory cytokines and apoptotic gene was observed in spleen of chicken infected by a velogenic AAvV-1, while minimal upregulation was observed by lentogenic isolates (Liu *et al*., 2012; Rajasekaran *et al*., 2018)

Previous studies reported isolation of virulent NDV from healthy village chicken (Ananth *et al*., 2008; Munir *et al*., 2012). In this study, for the first time, we confirm circulation of virulent AAvV-1 in apparently healthy emu birds. Emus exhibit certain level of indigenous resistance which might have helped the birds to withstand ND infection without exhibiting clinical signs, but they may act as carriers and transmit infection to poultry. AAvVs that spill-over from wild/feral birds or other species into poultry continue to evolve as they are rapidly passaged in chickens. Hence, extensive studies are required to estimate the prevalence of AAvV-1 in emu and their role in epidemiology and transmission of AAvV-1 in domestic poultry.

CONCLUSION

Better understanding on the epidemiological relations among the circulating AAvV-1, their genetic diversity, role of domesticated birds other than chicken in the maintenance and transmission of virus is quite essential and crucial for planning novel control strategies for ND.

ACKNOWLEDGEMENT

We are very thankful to the Sri Venkateswara Veterinary University (SVVU), Tirupati for providing facilities to carry out the research.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Alexander, D.J., Manvell, R.J. and Parsons, G. (2006). Newcastle disease virus strain Herts 33/56 in tissues and organs of chickens infected experimentally. Avian Pathology. 35(2): 99-101.

Ananth, R., Kirubaharan, J.J., Priyadarshini, M.L.M. and Albert, A. (2008). Isolation of Newcastle disease viruses of high virulence in unvaccinated healthy village chickens in south India. International Journal of Poultry Science. 7: 368-373.

Culling, C.F.A. (1968). Histological and Photochemical Staining Techniques, 3rd ed, Woods Worth Publication Pvt. Ltd., London.

Dimitrov, K.M., Abolnik, C., Afonso, C.L., Albina, E., Bahl, J., Berg, M., Briand, F.X., Brown, I.H., Choi, K.S., Chvala, I. and Diel, D.G. (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infection, Genetics and Evolution. 74: 103917.

Freshney, R.I. (2015). Culture of Animal Cells: A Manual of Basic Technique and Specialized Aplications. John Wiley and Sons.

Gowthaman, V., Ganesan, V., Gopala Krishna Murthy, T.R., Nair, S., Yegavinti, N., Saraswathy, P.V., Suresh Kumar, G., Udhayavel, S., Senthilvel, K. and Subbiah, M. (2018). Molecular phylogenetics of Newcastle disease viruses isolated from vaccinated flocks during outbreaks in Southern India reveals circulation of a novel sub genotype. Transboundary and Emerging Diseases. 66(1): 363-372.

Gowthaman, V., Singh, S.D., Dhama, K., Desingu, P.A., Kumar, A., Malik, Y.S. and Munir, M. (2016). Isolation and characterization of genotype XIII Newcastle disease virus from Emu in India. Virus Disease. 27(3): 315-318.

Jakhesara, S.J., Prasad, V.V.S.P., Pal, J.K., Jhala, M.K., Prajapati, K.S. and Joshi, C.G. (2016). Pathotypic and sequence characterization of Newcastle disease viruses from vaccinated chickens reveals circulation of genotype II, IV and XIII and in India. Transboundary and Emerging Diseases. 63(5): 523-539.

Kaleta, E.F. and Baldauf, C. (1988). Newcastle Disease in Free-Living and Pet Birds. In: Newcastle Disease. Springer, Boston, MA. pp 197-246.

Kinde, H., Hullinger, P.J., Charlton, B., McFarland, M., Hietala, S.K., Velez, V., Case, J.T., Garber, L., Wainwright, S.H., Mikolon, A.B. and Breitmeyer, R.E. (2005). The isolation of exotic Newcastle disease (END) virus from non poultry avian species associated with the epidemic of END in chickens in southern California: 2002-2003. Avian Diseases. 49(2): 195-198.

King, D.J. (1996). Influence of chicken breed on pathogenicity evaluation of velogenic neurotropic Newcastle disease virus isolates from cormorants and Turkeys. Avian Diseases. 1: 210-217.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution. 35(6): 1547-1549.

Liu, W.Q., Tian, M.X., Wang, Y.P., Zhao, Y., Zou, N.L., Zhao, F.F., Cao, S.J., Wen, X.T., Liu, P. and Huang, Y. (2012). The different expression of immune-related cytokine genes in response to velogenic and lentogenic Newcastle disease viruses infection in chicken peripheral blood. Molecular Biology Reports. 39(4): 3611-3618.

MC Ginnes, L., Sergel, T., Reitter, J. and Morrison, T. (2001). Carbohydrate modifications of the NDV fusion protein heptad repeat domains influence maturation and fusion activity. Virology. 283(2): 332-342.

Munir, M., Abbas, M., Khan, M.T., Zohari, S. and Berg, M. (2012). Genomic and biological characterization of a velogenic Newcastle disease virus isolated from a healthy backyard poultry flock in 2010. Virology Journal. 9(1): 46. http://www.virologyj.com/content/9/1/46.

Nantha Kumar, T., Kataria, R.S., Tiwari, A.K., Butchaiah, G. and Kataria, J.M. (2000). Pathotyping of Newcastle disease viruses by RT-PCR and restriction enzyme analysis. Veterinary Research Communications. 24(4): 75-86.

Office International des Epizooties (OIE). (2012). Newcastle disease. Manual of diagnostic tests and vaccines for terrestrial animals: Mammals, birds and bees. Biological Standards Commission. World Organisation for Animal Health. Paris, France, pp. 555-574.

Rajasekaran, R., Kirubaharan, J.J., Vidhya, M., Shilpa, P., Chandran, N. and Joy, D. (2018). Pro-inflammatory cytokine and apoptotic gene mRNA levels against lentogenic and velogenic Newcastle disease virus pathotypes in *in vivo* and *in vitro* biological systems. Indian Journal of Animal Research. 53(4): 515-522.

Shinde, P.V., Koratkar, S.S., Pawar, S.D., Kale, S.D., Rawankar, A.S. and Mishra, A.C. (2012). Serologic evidence of avian influenza H9N2 and paramyxovirus type 1 infection in emus (*Dromaius novaehollandiae*) in India. Avian Diseases. 56: 257-260.

Taxonomy, I.V. (2018). Release. (2019). International Committee on Taxonomy of Viruses.

Wajid, A., Dimitrov, K.M., Wasim, M., Rehmani, S.F., Basharat, A., Bibi, T., Arif, S., Yaqub, T., Tayyab, M., Ababneh, M. and Sharma, P. (2017). Repeated isolation of virulent Newcastle disease viruses in poultry and captive non-poultry avian species in Pakistan from 2011 to 2016. Preventive Veterinary Medicine. 142: 1-6.