Isolation and characterization of genotype XIII Newcastle disease virus from Emu in India

Vasudevan Gowthaman^{1,4} · Sambhu Dayal Singh¹ · Kuldeep Dhama¹ ·
Perumal Aurumugam Desingu¹ · Asok Kumar¹ · Yashpal Singh Malik² ·
Muhammad Munir³

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Abstract Newcastle disease virus (NDV) infects at least 241 species of pet and free-living birds in addition to domesticated avian species. Wild, feral and domesticated birds are recognized reservoirs of NDV, and contribute to the epidemiology of NDV in the domesticated poultry. The biological and molecular characterization of velogenic NDV (vNDV) from emus is limited. In this study, 54 tissues were collected from eight Emu flocks between May 2010 and January 2012 from highly poultry-dense areas of India including Namakkal, Hyderabad and Bareilly regions. The presence of vNDV was confirmed by virus isolation, fusion (F)-gene based RT-PCR, sequencing of the cleavage site and the virulence scored. One out of eight flocks received from Hyderabad region was found positive for NDV and the *in vivo* pathotyping revealed the isolate to be vNDV type. The sequence analysis revealed that the isolate had cleavage site of 112-R-R-R-K-R-F-117, which is typical for vNDV. Sequence and phylogenetic analysis of the partial ‘F’ gene coding regions suggested that the NDV strain belongs to genotype XIII. The Emu isolate had 98–100 % nucleotide identity with the vNDVs previously

reported in poultry flocks of India. The findings of present study based on the biological and molecular characterization of Emu-origin vNDV, highlights the circulation of genotype XIII in Emus for the first time in the country. There is need to understand the possible spill over of these genetically diverse NDV strains into the commercial poultry and their possible implications in disease control strategies.

Keywords Newcastle disease virus · Velogenic · Emu · India

Poultry farming is the one of the rapidly developing sectors plays an important role in global food security. The sustainability of this sector is continuously threatened by emergence of viral pathogens. Among the emerging diseases of poultry Newcastle disease (ND) is one of the most devastating diseases around the world. The etiological agent of ND is avian paramyxovirus type-1 (APMV-1), a member of the genus *Avulavirus* of the subfamily *Paramyxovirinae* in the *Paramyxoviridae* family under the order *Mononegavirales* [11, 13, 16]. In addition to domestic avian species, natural or experimental infections with ND virus (NDV) has been demonstrated in at least 241 species in 27 orders, and it is highly probable that all birds species are susceptible to NDV [8]. Based on the virus virulence, all strains of NDV are categorized into three major pathotypes viz., lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (high virulence). The velogenic NDV strains are further classified into either neurotropic or viscerotropic.

All NDV strains belong to a single serotype (i.e. APMV-1), however, there are large genetic and antigenic diversities among different isolates of NDV. Based on complete genome or the 3′ hypervariable region of the fusion protein (F) gene sequences, NDV isolates are classified into class I

✉ Kuldeep Dhama
kdhama@rediffmail.com

¹ Avian Diseases Section, Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India

² Division of Biological Standardization, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India

³ The Pirbright Institute, Woking, Surrey GU24 0NF, UK

⁴ Present Address: Poultry Disease Diagnosis and Surveillance Laboratory, Veterinary College and Research Institute Campus, Tamil Nadu Veterinary and Animal Sciences University, Namakkal 637 002, India

and class II main groups. Isolates belonging to class I are avirulent and are isolated from waterfowl and shorebirds, and occasionally from samples collected in live bird markets or from captured wild birds. Class II isolates consisting of both virulent and avirulent strains are present in both wild bird and poultry species; however, most virulent NDV isolates are obtained from poultry and are responsible for significant economic losses in the poultry industry worldwide. Currently, class I strains represent a single genotype, whereas class II strains represent at least 18 genotypes (I to XVIII) and several sub-genotype [3, 13, 16].

Wild, feral birds and domestic birds, except ducks and pigeons, are recognized as reservoirs of NDVs, and probably contribute to the epidemiology of ND in the domesticated poultry [2]. It has been suggested that the NDVs that spill-over from wild/feral birds or other species into poultry continue to evolve as they are rapidly passaged in chickens [4, 12]. Emu birds are also susceptible to many diseases including ND which infects domesticated poultry. Currently, there is limited information available on occurrence and characterization of APMV-1 in Emus [7, 10, 15]. The present study describes the isolation, and biological and genetic characterization of NDV strain from Emus.

A total of 54 pooled tissue samples consisting of trachea, spleen, intestine and kidney of moribund or dead birds were collected from eight Emu flocks had mortality of 0.4–2.5 % between May 2010 and January 2012 from high poultry dense areas of India including Hyderabad, Namakkal and Bareilly regions of Andhra Pradesh, Tamil Nadu, and Uttar Pradesh states respectively. Tissues from each flock were considered as a separate pool.

The tissues were processed (10 % homogenate in PBS) and inoculated into 9–11 days-old specific pathogen free (SPF) embryonated chicken eggs (Venkateshwara Hatcheries Private Limited, Pune, India) through allantoic route and eggs were incubated at 37 °C for a maximum period of 5 days and chilled for overnight at 4 °C. The embryos were candled every day and the dead embryos were chilled, and the allantoic fluids were used to determine the hemagglutination (HA) activity [14]. The presence of NDV in allantoic fluid was confirmed by HI using LaSota specific serum and RT-PCR assay. Three blind passages were carried out before deciding the negativity of the samples.

The virulence level of isolate was measured by means of intracerebral pathogenicity index (ICPI) in day-old chicks [1]. SPF eggs (Venkateshwara Hatcheries Private Limited, Pune, India) were hatched from isolated hatchery unit of Central Avian Research Institute (CARI), Izatnagar, India used for ICPI. All the experiments were carried out as per the guidelines of the Institutional Animal Ethics Committee and the Committee for the Purpose of Supervision and Control of Experiments on Animals, Ministry of

Environment and Forests, government of India. As part of this process the work has been approved by the ethics committee at the Indian Veterinary Research Institute, India.

Total RNA was extracted from allantoic fluid with the use of the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, California, USA) and reverse transcribed with Superscript First Strand Synthesis RT-PCR kit (Invitrogen, USA) and random primers. The presence NDV was confirmed by PCR targeting F gene [18] and virulence was determined by partial sequencing of F gene covering cleavage site.

The NDV F gene sequence was initially analyzed in ChromasPRO v1.7.3 (<http://technelysium.com.au/>) to confirm the quality of read data. After alignment, the sequence of the NDV isolates was subjected to blast analysis in NCBI BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic analysis based on the partial nucleotide sequence of F gene in the highly variable region including cleavage site was performed in MEGA6 software by using the Maximum Likelihood method with default settings and 1000 bootstrap re-sampling [17]. NCBI BLAST was used for nucleotide and amino acid identity comparisons between representative isolates within identified clusters.

The NDV infection was confirmed in one out of eight Emu flocks by using virus isolation and RT-PCR assays. The partial length (300 bp) F gene was sequenced and deposited in the GenBank (Accession Number: HG780861). The spleen and intestine samples of the particular Emu flock were received from Hyderabad region from dead Emus, without any known flock history. In vivo pathotyping confirmed that isolate belong to the velogenic type of NDV and carry an ICPI value of 1.76. Fusion protein gene sequence analysis revealed that the isolate had cleavage site of ¹¹²R-R-R-K-R/F¹¹⁷ which is characteristic of virulent strains, supporting the in vivo pathotyping results. Genetic comparison of the Emu isolate with the previously characterized Indian isolates circulating in commercial poultry revealed a high (98–100 %) homology. Phylogenetic analysis, conducted on the Emu NDV strain with the represented strain of all recognized genotypes, indicated that at least 13 representative genotypes could be clearly identified. Moreover, the genotype XIII can clearly be divided into two sub-genotypes; XIIIa and XIIIb. Clustering pattern and topology of the tree indicated the grouping of Emu strain of NDV, to genotype XIII strains, and more specifically into the sub-genotype XIIIb (Fig. 1).

The findings of present study confirm the circulation of velogenic NDV (vNDV) in Indian Emus. Previous results have demonstrated that infection of Emus with a Roakin strain of NDV failed to produce clinical disease or shed virus [6], indicating that although Emus are susceptible, they might show a certain level of indigenous resistance.

Fig. 1 The phylogenetic analysis based on the 300 bp nucleotide sequence of F gene in the highly variable region including F gene cleavage site. Tree was generated in the MEGA6 software using the Maximum Likelihood method based on the General Time Reversible model, and Gamma distributed with invariable sites (G + I)

Kinde and others [9] reported isolation of Exotic Newcastle disease (END) Virus from two emus with the epidemic of END in Chickens in Southern California during 2002–2003. These reports highlight the possible roles of Emu birds in the NDV pathobiology, however, the genetic characterization of vNDV and the biological roles of Emu in the epizootiology of NDV is limited especially in natural endemic situation. The NDV strain characterized in this study was collected from a naturally infected Emu in the field-conditions. Results demonstrated that the ICPI value correlated with the multiple basic amino acids in the F0 cleavage site ¹¹²R-R-R-K-R-F¹¹⁷. NDVs with an ICPI value >0.7 and/or presence of polybasic amino acids at its F0 cleavage site are considered virulent [14]. The isolate characterized herein showed an ICPI value of 1.76, indicating that the NDV circulating in Emu belongs to velogenic type, which could possess severe threat to the poultry industry. Phylogenetic analysis of the partial F coding regions of current Emu isolate suggests that Indian Emu NDV belongs to class II genotype XIII and subgenotype XIIIb. The Emu NDV isolate shared 98–100 % identity with vNDV sequences isolated from domesticated poultry in the same period, suggesting that same vNDVs might have circulated in Emus and chicken [5]. Furthermore, the confirmation of vNDV in an Emu flock raised in a poultry dense area, hypothesize the potential role of Emus in the transmission of vNDVs. In Indian field conditions Emus are housed in open pens, which provide opportunities for interaction with other wild and feral birds. Although, it has been demonstrated that as little as 15.3 % of Emus are seropositive in some regions of India [15], extensive studies are required to estimate the prevalence of NDV in Emu and their role in epidemiology and transmission of NDV in domestic poultry.

In conclusion, we report the first characterization and confirm the circulation of vNDV of genotype XIII in Indian emus. It is potentially plausible that Emu might contribute in the spread of the currently circulating NDV genotype XIII in domesticated poultry. Further extensive surveillance is needed to understand the epidemiology of NDV in the country which will not only help to assess the roles of Emu in disease emergence but would also facilitate the selection of effective control measures to prevent the disease in birds.

References

- Alexander DJ. Newcastle disease and other avian Paramyxovirus. In: Swayne D, Glisson JR, Jackwood MW, Pearson JE, Reed WM, editors. A laboratory manual for the isolation and identification of avian pathogens. 4th ed. Kennett Sq.: Am. Assoc. Avian Pathol; 1998. p. 156–63.
- Alexander DJ. Newcastle disease in the European Union 2000 to 2009. Avian Pathol J WVPA. 2011;40:547–58.
- Diel G, da Silva LHA, Liu H, Wang Z, Miller PJ, Afonso CL. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis. 2012;12:1770–9.
- Garcia SC, Lopez RN, Morales R, Olvera MA, Marquez MA, Merino R, et al. Molecular epidemiology of Newcastle disease in Mexico and the potential spillover of viruses from poultry into wild bird species. Appl Environ Microbiol. 2013;79:4985–92.
- Gowthaman V. Etio-Pathology and differential diagnosis of low pathogenic avian influenza (LPAI) in poultry. PhD Thesis, Indian Veterinary Research Institute 2011.
- Heckert RA, McIsaac M, Chan M, Zhou EM. Experimental infection of emus (*Dromaius novaehollandiae*) with avian influenza viruses of varying virulence: clinical signs, virus shedding and serology. Avian Pathol J WVPA. 1999;28:13–6.
- Jørgensen PH, Herczeg J, Lomniczi B, Manvell RJ, Holm E, Alexander DJ. Isolation and characterization of avian paramyxovirus type 1 (Newcastle disease) viruses from a flock of ostriches (*Struthio camelus*) and emus (*Dromaius novaehollandiae*) in Europe with inconsistent serology. Avian Pathol J WVPA. 1998;27:352–8.
- Kaleta E, Baldauf C. Newcastle disease in free-living and pet birds. In: Alexander DJ, editor. Newcastle disease. Boston: Kluwer; 1998. p. 197–246.
- Kinde H, Hullinger PJ, Charlton B, McFarland M, Hietala SK, Velez V, et al. The isolation of exotic Newcastle disease (END) virus from nonpoultry avian species associated with the epidemic of END in chickens in southern California: 2002–2003. Avian Dis. 2005;49:195–8.
- Kumanan K, Meignanalakshmi A, Meenu R, Nainar AM. Pathotyping of a Newcastle disease virus isolated from Emus (*Dromaius novaehollandiae*). Trop Anim Health Prod. 2003;35:391–5.
- Mayo MA. A summary of taxonomic changes recently approved by ICTV. Arch Virol. 2002;147:1655–63.
- Miller PJ, Decanini EL, Afonso CL. Newcastle disease: evolution of genotypes and the related diagnostic challenges. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis. 2010;10:26–35.
- Miller PJ, Haddas R, Simanov L, Lublin A, Rehmani SF, Wajid A, et al. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis. 2015;29:216–29.
- OIE. Newcastle Disease. Man Diagn Tests Vaccines Terr Anim. Chapter 2.3.14. 2012.
- Shinde PV, Koratkar SS, Pawar SD, Kale SD, Rawankar AS, Mishra AC. Serologic evidence of avian influenza H9N2 and paramyxovirus type 1 infection in emus (*Dromaius novaehollandiae*) in India. Avian Dis. 2012;56:257–60.
- Susta L, Jones MEB, Cattoli G, Cardenas-Garcia S, Miller PJ, Brown CC, et al. Pathologic characterization of genotypes XIV and XVII Newcastle disease viruses and efficacy of classical vaccination on specific pathogen-free birds. Vet Pathol. 2015;52:120–31.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.
- Toyoda T, Sakaguchi T, Hirota H, Gotoh B, Kuma K, Miyata T, et al. Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. Virology. 1989;169:273–82.