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Title: **Molecular phylogenetics of Newcastle disease viruses isolated from vaccinated flocks during outbreaks in Southern India reveals circulation of a novel sub-genotype**

Running title: **Novel sub-genotype XIIIe NDV in India**

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SUMMARY

Newcastle disease (ND) is an economically important, contagious poultry viral disease reported across the globe. In India, ND is endemic and episodes of ND outbreaks despite strict vaccinations are not uncommon. We isolated and characterized seven ND viruses from vaccinated commercial poultry farms during severe disease outbreaks in Tamil Nadu, in Southern India, between April 2015 and June 2016. All the seven isolates were categorized as virulent by mean death time (48-54 hrs) in embryonated chicken eggs. Also, their sequences carried the virulence signature of multi-basic amino acid residues in their fusion protein cleavage site (RRQ/RR/KRF). Phylogenetic and evolutionary distance analyses revealed circulation of a novel sub-genotype of genotype XIII, class II ND viruses, herein proposed as sub-genotype XIIIe. The genetic divergence between the circulating virulent strains and the vaccine strains could possibly explain the disease outbreak in the vaccinated flocks. Further, our study signifies the need to implement routine epidemiological surveillance and to revisit the current vaccination program.

KEY WORDS:

Newcastle disease virus, genotype XIII, sub-genotype XIIIe, fusion protein, phylogenetic analyses and evolutionary distance estimation

1 INTRODUCTION

Newcastle disease virus (NDV) is an economically important, contagious poultry pathogen that causes respiratory, nervous and enteric Newcastle disease (ND). NDV is known to infect more than 236 avian species and is spread across the globe (Miller, Decanini, & Afonso, 2010). NDV is an enveloped virus belonging to serotype 1 of avian paramyxoviruses grouped

in the genus *Avulavirus* within family *Paramyxoviridae* (Alexander, 1998). NDV has a negative-sense, single-stranded, non-segmented RNA genome. The RNA genome is approximately 15 kb and carries six genes arranged in tandem each coding for a single protein, namely, nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), hemagglutinin-neuraminidase glycoprotein (HN) and large polymerase protein (L) (Alexander, 2000). Additionally, by co-transcriptional editing of P gene, two non-structural proteins, V and W, are expressed during viral infections (Steward, Vipond, Millar, & Emmerson, 1993).

Among the viral proteins, F glycoprotein is involved in fusion of viral and cell membranes and has been identified as one of the key determinants of viral pathogenicity (Paldurai et al, 2014). F protein is cleaved by host proteases to F1 and F2 polypeptides linked by disulphide bonds. The presence of multi-basic amino acid residues at the fusion protein cleavage site determines the virulence of NDV or pathotypes (Hanson & Brandly, 1955). NDV strains are grouped into three pathotypes: (i) lentogenic or avirulent strains (ii) mesogenic or moderately virulent strains and (iii) velogenic or highly virulent strains, depending on their virulence determined by mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI). MDT is the mean time in hours for the minimum lethal dose of the virus required to kill all the inoculated chicken embryos. Velogenic NDV strains take less than 60 hours, mesogenic strains take between 60 and 90 hours and lentogenic strains take more than 90 hours to kill the inoculated embryos (Alexander, 1998). According to definitions published by the World Organization for Animal Health (OIE), highly virulent NDV has either an ICPI of 0.7 in day-old chicks when injected intracerebrally and observed for 8 days for sickness or death, or carries multiple basic amino acids, at least three arginine (R) or lysine (K) residues at fusion protein cleavage site starting at amino acid position 113, and a phenylalanine residue at position 117 (OIE, 2012).

Based on their fusion protein sequences, NDV strains are classified into two classes, I and II. All class I strains belong to a single genotype. These viruses were isolated from waterfowls, shorebirds and live bird market samples and are found to be avirulent in chickens (Ramey et al, 2013; Lindh et al, 2014; Zhang et al, 2015). The class II strains are categorised into eighteen genotypes based on their genetic differences and include avirulent, mesogenic and velogenic strains (Kim et al, 2007; Miller, Kim, Ip, & Afonso, 2009; Miller, Decanini, & Afonso, 2010; Courtney et al, 2013; de Almeida et al, 2013; Snoeck et al, 2013). NDV strains are further classified into sub-genotypes within genotypes based on their inter-population evolutionary distances (Diel, da Silva, Liu, Wang, Miller, & Afonso, 2012).

NDV is endemic in India and reports of frequent disease outbreaks are common in spite of strict vaccinations (Jakhesara, Prasad, Pal, Jhala, Prajapati, & Joshi, 2016). There are reports of disease outbreak caused by circulating genotype XIII viruses of class II NDV in vaccinated flocks (Ghodasara et al, 2015). It must be noted here that the strains used in currently-available vaccines against NDV (strains such as VG/GA, F, LaSota,R2B, Kumarov and Mukteswar) belong to genotype II (Miller et al, 2010). The most ancestral strain of genotype XIII was isolated in India from a cockatoo bird in 1982 (Benson, Clark, Karsch-Mizrachi, Lipman, Ostell, & Sayers, 2015). It is now known that genotype XIII viruses are currently prevalent in India and there are reports of circulation of genotype XIII variants (Gowthaman et al, 2016; Nath & Kumar, 2017). All the viruses belonging to genotype XIII are virulent and mostly isolated from chickens. Spread of infection from poultry to wild birds has also been recorded (Usachev, Fediakina, Shchelkanov, Vov, Prilipov, & Iamnikova, 2006). To date four sub-genotypes of genotype XIII viruses, XIIIa, XIIIb, XIIIc and XIIId, have been identified (Dimitrov, Ramey, Qiu, Bahl, & Afonso, 2016; Mayahi & Esmaelizad, 2017; Nath & Kumar, 2017). Sub-genotype XIIIa circulates in Europe, Asia, Africa and the Middle East and sub-genotype XIIIb viruses are documented in Asia, specifically in India and in Pakistan

(Khan et al, 2010; Ebrahimi, Shahsavandi, Moazenijula, & Shamsara, 2012; Munir, Abbas, Khan, Zohari, & Berg, 2012; Shabbir et al, 2012; Jakhesara et al, 2016; Benson, Clark, Karsch-Mizrachi, Lipman, Ostell, & Sayers 2015; Gogoi, Morla, Kaore, Kurkure, & Kumar, 2015). Sub-genotype XIIIc viruses were isolated from eastern and northeastern India (Nath & Kumar, 2017). Mayahi and Esmaelizad recently, reassigned a group of sub-genotype XIIIa viruses, isolated between 2008 and 2011 in Iran, as sub-genotype XIIId owing to their evolutionary divergence values. These reports suggest genotype XIII viruses are evolving rapidly.

Knowledge of the circulating NDV strains is essential for taking measures to prevent epidemics of ND, which is a constant threat to poultry industry in India. Also it has huge impact on the socio-economic developments of the Indian farming community which contribute to 21% of total egg production through backyard poultry farming alone. In this study, we isolated and characterized seven viruses from ND outbreaks in NDV vaccinated flocks in commercial poultry farms in southern part of India between April 2015 and June 2016. Based on their complete fusion protein sequences, phylogenetic and evolutionary divergence data were generated to understand the cause of disease outbreak in vaccinated birds.

2 MATERIALS AND METHODS

2.1 Case history

Samples were collected from seven commercial poultry farms in Tamil Nadu (TN) state of India with clinical history suggesting respiratory/neurological disease during the period between April 2015 and June 2016. The vaccination record in the farm showed that all birds in the farm were vaccinated with ND clone vaccine at 6^{th} day, ND Killed vaccine at 15^{th} day, followed by ND LaSota at 8^{th} week of age in color broiler farms (PDDSL-3, -4, -5), in

addition to these vaccines the commercial layer chickens (PDDSL-1 & -2) were vaccinated with R2B at 11th week of age followed by ND killed at the point of lay. As per the history of the farm where PDDSL-6 was isolated, the farmer had vaccinated his birds with ND LaSota during 60 days of age. No vaccination records were available for the backyard poultry farm where PDDSL-7 was isolated. Tissue samples from ND suspected freshly dead birds were used for histopathology, virus isolation and characterization. Pooled tissue samples (trachea, spleen, kidney, oviduct and bursa) collected from at least 5 dead birds per farm were processed for viral isolation.

2.2 Virus isolation and propagation

Seven NDV were isolated and identified by standard laboratory procedures (Alexander, 1998). Briefly, the tissues were processed aseptically and inoculated into 9- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs through allantoic route. The eggs were incubated at 37°C until the embryos died or for a maximum period of 120 hours, whichever was earlier. The embryos were candled every day and the dead embryos were chilled at 4°C overnight for collection of allantoic fluid. Hemagglutination activity (HA) was performed with the allantoic fluid as described in the OIE manual (OIE, 2012). The presence of NDV in allantoic fluid was then confirmed by hemagglutination inhibition (HI) test using NDV LaSota specific serum (OIE, 2012) and further ascertained by reverse transcription-PCR using NDV consensus primers. Three blind passages were carried out before deciding virus negativity of the samples. The isolates were labeled as PDDSL-1 to -7 and their information is provided in Table 1.

2.3 RNA isolation, PCR amplification, and sequencing

Viral RNA from HA- positive allantoic fluid samples was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany). cDNA was synthesized using random primer and SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed using NDV F gene specific consensus primers (Table 2). The general conditions for PCR were 95°C for 5 min, 20 cycles of 95°C for 1 min (denaturation), 54°C for 1 min (annealing) and 72°C for 1 min (extension), followed by 72°C for 10 min (final extension). The complete F proteins of all the seven isolates were sequenced from the PCR products by Sanger sequencing and submitted to GenBank (GenBank IDs: MF362982, MF362983, MF362984, MF362985, MF362986, MF362987 and MF362988).

2.4 F protein sequence analysis: Phylogenetic relationships and Evolutionary divergence estimates:

The full-length F protein sequence data of all the seven NDV isolates were subjected to blast analysis using NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with commonly used NDV vaccine strains. The fusion protein sequences of 110 NDV sequences representing different genotypes and sub-genotypes including the seven isolates in this study (supplementary table 1) were aligned by Clustal W and annotated using MEGA7 software (www.megasoftware.net). Two phylogenetic trees were constructed, one with 110 nucleotide sequences of viruses belonging to 18 genotypes and the other with 58 nucleotide sequences of viruses belonging to genotype XIII alone , using maximum likelihood method by bootstrapping with 500 replications (Felsenstein, 1985) based on the Tamura-Nei model (Tamura and Nei, 1993). All positions containing gaps and missing data were eliminated. There were a total of 354 positions in the final dataset. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic

search were obtained automatically by applying Neighbor-Joining 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. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.6631)] and the evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

The evolutionary relationship between the seven isolates and 103 viruses belonging to the 18 genotypes of class II NDV were estimated by calculating the number of base differences per site from averaging over all sequence pairs between groups using MEGA7 (Kumar, Stecher, & Tamura, 2016).

3 RESULTS

3.1 Virus isolation and characterization

Information about the virus isolates PDDSL-1 to PDDSL-7 are provided in Table 1. All the affected birds showed typical clinico-pathological signs of Newcastle disease. All of the NDV isolates were classified as velogenic, with MDT values ranging from 48 to 54 hours.

3.2 Analysis of the fusion protein sequences of the isolates

The sequences of the complete F protein of all the seven isolates are currently available in GenBank and can be identified by MF362982, MF362983, MF362984, MF362985, MF362986, MF362987 and MF362988. All the isolates carried multi-basic amino acid residues at the fusion protein cleavage site, between amino acid positions 113 and 116 and a phenylalanine at position 117, typical of virulent strains (OIE, 2012). The fusion proteins of the isolates showed amino acid sequence identity of 88.6% to 91.5% with vaccine strain

Mukteswar, amino acid sequence identity of 86.3% to 89.5% with vaccine strain LaSota and amino acid sequence identity of 86.1% to 89.3% with vaccine strain B1. Table 3 shows the amino acid substitutions observed within heptad repeat regions, HRb, HRc regions and transmembrane domains. There was no change in the fusion peptide region. The amino acid residues in the hypervariable region, fusion protein cleavage site and neutralizing epitopes of F protein sequences of the isolates were compared with those of NDV vaccine strains LaSota, B1 and Mukteswar (Table 4). There were not many changes in the neutralizing epitopes. Only PDDSL-1 and PDDSL-2 viruses among the seven isolates showed amino acid differences at positions 75 (Alanine to Glutamic acid) and at 170 (Aspartic acid to Asparagine) when compared with vaccine strains LaSota and B1. Potential glycosylation sites with the N-X-T/S motif (in which X may be any amino acid except proline) were predicted by NetNGlyc version 1.0 and all the isolates carried the six glycosylation sites similar to other NDV strains.

3.3 Phylogenetic analysis

The phylogenetic analysis based on the full-length fusion nucleotide sequences classified all the seven isolates as members of genotype XIII of class II NDV. PDDSL-1, -2 and -6 viruses clustered closely with sub-genotype XIIIb viruses and PDDSL-3, -4, -5 and -7 viruses clustered together and are hereby designated into a novel sub-genotype, XIIIe (Figures 1 and 2). The evolutionary distance between the seven isolates and other NDV genotypes estimated by MEGA7 reconfirmed the grouping of PDDSL isolates within genotype XIII. The divergence data suggested that the isolates PDDSL-1 and -2 were closely related by a distance of 2.8 % with isolate PDDSL-6 and the sub-genotype XIIIb virus, NDV2K35/CH/TN/2003 (GenBank ID KF740478.1). Furthermore, the isolate PDDSL-6 was found closely related with the following sub-genotype XIIIb viruses: was identical with NDV

isolate/NDV2K35/CH/TN/2003 (GenBank ID KF740478.1), showed a distance of 1.1% with NDV52/SARSA/2014 (GenBank ID KM056350.1), showed a distance of 2.3% with following NDV isolates, NDV16/GODHRA/03/2013 (GenBank ID KM056344.1), NDV20/NAVLI/03/2013 (GenBank ID KM056345.1), NDV26/NAVLI/03/2013 (GenBank ID KM056346.1), NDV40/SARSA/04/2013 (GenBank ID KM056348.1), NDV42/GOPALPURA/04/2013 (GenBank ID KM056349.1), NDV/chicken/Nagpur/06/06 (GenBank ID KX372709.1), and showed a distance of 2.5% with NDV54/HYDERABAD (GenBank ID KM056352.1) and NDV/NAGPUR (GenBank ID KP089979.1). This data suggests circulation of genotype XIIIb viruses with lower divergence. The evolutionary distance estimates of the other four isolates, PDDSL-3, -4, -5 and -7 indicated a close relation between them (Table 5) but were highly divergent from other sub-genotypes of genotype XIII supporting their designation into the novel sub-genotype XIIIe (Table 6).

4 DISCUSSION

Seven viruses were isolated during ND outbreaks in poultry farms in South India between April 2015 and June 2016. All these poultry farms had routinely vaccinated their flocks against NDV using vaccine strains such as ND clone vaccine, LaSota, and R2B. NDV was detected from seven flocks with history of respiratory and neurological disease. The virus isolates were confirmed as NDV by HA, HI using NDV specific sera and PCR using NDV specific primers. Both MDT and F protein cleavage site sequences indicated that all the isolates are velogenic. The findings of the present study reconfirms the earlier reports of NDV isolation in association with respiratory and neurological diseases (Wakamatsu, King, Kapczynski, Seal, & Brown, 2006; Cattoli, Susta, Terregino, & Brown, 2011). The farms investigated were found to be often inhabited by the feral birds like crows, Indian mynahs and cattle egrets; these birds may have played a role in transmission of NDV as previously

reported (Roy, Venugopalan, Selvarangam, & Ramaswamy, 1998; Kinde et al, 2005; Terregino et al, 2013). The clinico-pathological findings observed in the NDV positive flocks were in agreement with the earlier studies of (Susta, Miller, Afonso, & Brown, 2011, Susta, Jones, Cattoli, Cardenas-Garcia, Miller, Brown, & Afonso 2015), which leads to speculation that the genotype XIII NDVs could be surpassing the protective antibody titres, undergoing extra intestinal replication leading to lesions in various organs. Further studies are required to demonstrate the virus replication in different tissues by IHC/In-Situ hybridization and by real time PCR.

Disease outbreaks by genotype XIII viruses in vaccinated flocks is not uncommon (Munir, Abbas, Khan, Zohari, & Berg, 2012; Ghodasara et al 2015; Zhu et al, 2016). Currently in India, avirulent strains such as VG/GA, F, D58, Clone 30, LaSota and mesogenic strains for example, Komarov and Mukteswar are used as vaccines against NDV infection; all of these belong to genotype II. The virus isolates in this study showed no difference in the neutralizing epitopes in F protein when compared with the vaccine strains. However, additional data from whole genome sequencing as well as from animal experiments are required to identify if there are any mutations in the genome that could lead to neutralization escape variants.

According to the new unified system for classification of NDVs there are two distinct classes of NDV, class I includes a single genotype, and class II comprises of 18 genotypes (Diel, da Silva, Liu, Wang, Miller, & Afonso, 2012; Miller et al, 2015; Dimitrov, Ramey, Qiu, Bahl, & Afonso, 2016). Further viruses within a genotype that show interpopulation evolutionary distance of more than 3% but less than 10% are designated as different sub-genotypes within a genotype (Diel, da Silva, Liu, Wang, Miller, & Afonso, 2012). Analysis of the fusion

protein sequences by phylogenetic tree using maximum likelihood method grouped the isolates of this study within genotype XIII viruses of class II NDV. Estimation of the evolutionary divergence of the seven isolates of this study from other strains of genotype XIII revealed a novel sub-genotype (herein proposed as sub-genotype XIIIe). The PDDSL-1, -2 and -6 viruses were grouped with sub-genotype XIIIb while PDDSL-3, -4, -5 and -7 isolates clustered together forming a novel sub-genotype XIIIe. The sub-genotype XIIIe reported here consists of four strains and is highly divergent from all the previously reported sub-genotypes of genotype XIII, sufficient to be termed as a novel sub-genotype (Miller et al., 2015). Our data also suggests that the sub-genotype XIIIe viruses could have emerged from sub-genotype XIIIb viruses. The results from this study further reiterates the continuous evolution of genotype XIII as reported recently (Barman et al, 2017; Mayahi & Esmaelizad, 2017).

The knowledge of the currently circulating viruses is necessary for developing effective recombinant vaccines as it is now becoming increasingly evident that antigenically matched vaccines can provide better immunity (Miller et al, 2013; Ji et al, 2018). On the other hand, one cannot also completely ignore factors other than the vaccine genotype, such as a break in cold chain and inefficient vaccination procedures leading to insufficient immunity and thus contributing to disease outbreak in the vaccinated flocks.

In conclusion, we report here circulation of novel sub-genotype XIIIe NDV in India. These viruses, isolated during disease outbreaks in vaccinated flocks are virulent and pose a threat to poultry production. Our data indicates constant evolutionary changes occurring in the circulating sub-genotype XIIIb viruses, highlights the importance of epidemiological

surveillance for undertaking effective disease control measures to prevent epidemics and urges the need to review the current vaccination program.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHOR CONTRIBUTIONS:

V.G[1] and M.S conceived the study. V.G[1], G.K.M, G.S.K, S.U, K.S collected the samples, isolated the viruses and characterized them. M.S supervised the study involving sequencing, designed the experiments and interpreted the data. S.N, N.Y, S.P.V performed the sequencing experiments. V.G[2] and M.S analysed the sequences. M.S and V.G[1] prepared the manuscript and all authors have read and approved the manuscript.

ETHICAL APPROVALS:

This study did not involve any human or animal subjects. For handling the samples and viruses at TANUVAS appropriate biosafety measures were followed. Institutional biosafety guidelines were followed at NIAB to handle the RNA and cDNA samples obtained from TANUVAS for this study.

REFERENCES:

Alexander, D.J.(1998). Newcastle disease virus and other avian paramyxoviruses. Laboratory Manual for Isolation and Identification of Avian Pathogens. Ed Swayne Glisson JR Jackwood MW Pearson JE Reed WM 4th Ed Am. Assoc. Avian Pathol. Kennett Sq. PA. pp. 156–163.

Alexander, D.J. (2000). Newcastle disease and other avian paramyxoviruses. *Revue scientifique et technique*, 19, 443–462.

Barman, L.R., Nooruzzaman, M., Sarker, R.D., Rahman, M.T., Saife, M.R.B., Giasuddin, M., … Islam, M.R. (2017). Phylogenetic analysis of Newcastle disease viruses from Bangladesh suggests continuing evolution of genotype XIII. *Archives of Virology,* 162, 3177-3182.

Benson, D.A., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., & Sayers E.W. (2015). GenBank. *Nucleic Acids Research*, 43, D30–35.

Cattoli, G., Susta L., Terregino, C., & Brown, C. (2011). Newcastle disease: a review of field recognition and current methods of laboratory detection. *Journal of Veterinary Diagnostic Investigation*, 23, 637–656.

Courtney, S.C., Susta, L., Gomez, D., Hines, N.L., Pedersen, J.C., Brown, C.C., …Afonso, C.L. (2013). Highly divergent virulent isolates of Newcastle disease virus from the Dominican Republic are members of a new genotype that may have evolved unnoticed for over 2 decades. *Journal of Clinical Microbiology*, 51, 508–517.

Das, M., & Kumar, S. (2017). Evidence of independent evolution of genotype XIII Newcastle disease viruses in India. *Archives of Virology*, 162, 997–1007.

De Almeida, R.S., Hammoumi, S., Gil, P., Briand, F.X., Molia, S., Gaidet, N., … Albina, E. (2013). New avian paramyxoviruses type I strains identified in Africa provide new outcomes for phylogeny reconstruction and genotype classification. *PloS One*, 8, e76413.

Diel, G., da Silva, L.H.A., Liu, H., Wang, Z., Miller, P.J. & Afonso, C.L. (2012). Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infection, Genetics and Evolution,* 12, 1770–1779.

Dimitrov, K.M., Ramey, A.M., Qiu, X., Bahl, J., & Afonso, C.L. (2016). Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). *Infection, Genetics and Evolution,* 39, 22–34.

Ebrahimi, M.M., Shahsavandi, S., Moazenijula, G., & Shamsara, M. (2012). Phylogeny and evolution of Newcastle disease virus genotypes isolated in Asia during 2008-2011. *Virus Genes*, 45, 63–68.

Felsenstein, J. (1985). Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*. 39, 783–791.

Ghodasara, P.D., Joshi, B.P., Prajapati, K.S., Ghodasara, D.J., Mathakiya R.A., Khorajiya, J.H., & Pandey, S. (2015). Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. *Veterinary World*, 8, 372–381.

Gogoi, P., Morla, S., Kaore, M., Kurkure, N.V., & Kumar, S. (2015). Complete genome sequence of a newcastle disease virus isolate from an outbreak in central India. *Genome Announcement*, 3, e01418-14.

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

Hanson, R.P., & Brandly, C.A. (1955). Identification of vaccine strains of Newcastle disease virus. *Science*, 122, 156–157.

Jakhesara, S.J., Prasad, V.V.S.P., Pal, J.K., Jhala, M.K., Prajapati, K.S., & 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, 523–539.

Ji, Y., Liu, T., Du, Y., Cui, X., Yu Q., Wang, Z., … Zhu, Q. (2018). A novel genotype VII Newcastle disease virus vaccine candidate generated by mutation in the L and F genes confers improved protection in chickens. *Veterinary Microbiology*, 216, 99–106.

Kaleta, E., & Baldauf, C. (1998). Newcastle disease in free-living and pet birds. DJ Alexander Ed Newctle Disease. Pp 197–246 Boston MA Kluwer Acad. Publ.

Khan, T.A., Rue, C.A., Rehmani, S.F., Ahmed, A., Wasilenko, J.L., Miller, P.J.& Afonso, C.L. (2010). Phylogenetic and biological characterization of Newcastle disease virus isolates from Pakistan. *Journal of Clinical Microbiology*, 48, 1892–1894.

Kim, L.M., King, D.J., Suarez, D.L., Wong, C.W. & Afonso, C.L. (2007). Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR. *Journal of Clinical Microbiology*, 45, 1310–1314.

Kinde, H., Hullinger, P.J., Charlton, B., McFarland, M., Hietala, S.K., Velez, V., … Ardans, A.A. (2005). 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 Diseases*, 49, 195–198.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33, 1870–1874.

Lindh, E., Ek-Kommonen, C., Väänänen, V. M., Vaheri, A., Vapalahti, O., & Huovilainen, A. (2014). Molecular epidemiology of H9N2 influenza viruses in Northern Europe. *Veterinary Microbiology*, 172, 548–554.

Mayahi, V., & Esmaelizad, M. (2017). Molecular evolution and epidemiological links study of Newcastle disease virus isolates from 1995 to 2016 in Iran. *Archives of Virology*, 162, 3727–3743.

Miller, P.J., Afonso, C.L., El Attrache, J., Dorsey, K.M., Courtney, S.C., Guo, Z. & Kapczynski, D.R. (2013). Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. *Developmental and Comparative Immunology*, 41, 505–513.

Miller, P.J., Decanini, E.L., & Afonso, C.L. (2010). Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infection, Genetics and Evolution*, 10, 26–35.
Miller, P.J., Haddas, R., Simanov, L., Lublin, A., Rehmani, S.F., Wajid, A., … Afonso, C.L. (2015). Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infection, Genetics and Evolution, 29, 216–229.
Miller, P.J., Kim, L.M., Ip, H.S., & Afonso, C.L. (2009). Evolutionary dynamics of Newcastle disease virus. *Virology*, 391, 64–72.
Munir, M., Abbas, M., Khan, M.T., Zohari, S., & 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, 46, 1-11.
Nath, B., & Kumar, S. (2017). Emerging variant of genotype XIII Newcastle disease virus from Northeast India. *Acta Tropica*, 172, 64–69.
OIE. (2012). Newcastle Disease. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.3.14.1-20.
Paldurai, A., Kim, S. H., Nayak, B., Xiao, S., Shive, H., Collins, P.L. & Samal, S.K. (2014). Evaluation of the contributions of individual viral genes to newcastle disease virus virulence and pathogenesis. *Journal of Virology*, 88, 8579–8596.
Ramey, A.M., Reeves, A.B., Ogawa, H., Ip, H.S., Imai, K., Bui, V.N., … Afonso, C.L. (2013). Genetic diversity and mutation of avian paramyxovirus serotype 1 (Newcastle disease virus) in wild birds and evidence for intercontinental spread. *Archives of Virology*. 158, 2495–2503.
Roy, P., Venugopalan, A.T., Selvarangam, R., & Ramaswamy, V. (1998). Velogenic Newcastle disease virus in captive wild birds. *Tropical Animal Health and Production*, 30, 299–303.
Shabbir, M., Goraya, M., Abbas, T., Yaqub, M., Shabbir, M., Ahmad, M.,… Munir, M. (2012). Complete genome sequencing of a velogenic viscerotropic avian paramyxovirus 1 isolated from pheasants (Pucrasia macrolopha) in Lahore. *Pakistan Journal of Virology*, 86, 13828–13829.
Snoeck, C.J., Marinelli, M., Charpentier, E., Sausy, A., Conzemius, T., Losch, S., & Muller, C.P. (2013). Characterization of newcastle disease viruses in wild and domestic birds in Luxembourg from 2006 to 2008. *Applied and Environmental Microbiology*. 79, 639–645.
Steward, M., Vipond, I.B., Millar, N.S.& Emmerson, P.T. (1993). RNA editing in Newcastle disease virus. *Journal of General Virology*,74, 2539–2547.
Susta, L., Jones, M.E.B., Cattoli, G., Cardenas-Garcia, S., Miller, P.J., Brown, C.C., & Afonso, C.L. (2015). Pathologic characterization of genotypes XIV and XVII Newcastle disease viruses and efficacy of classical vaccination on specific pathogen-free birds. *Veterinary Pathology*, 52, 120–131.
Susta, L., Miller, P.J., Afonso, C.L., & Brown, C.C. (2011). Clinicopathological characterization in poultry of three strains of Newcastle disease virus isolated from recent outbreaks. *Veterinary Pathology*, 48, 349–360.
Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*. 10, 512–526.
Terregino, C., Aldous, E.W., Heidari, A., Fuller, C.M., De Nardi, R., Manvell, R.J., … Capua, I. (2013). Antigenic and genetic analyses of isolate APMV/wigeon/Italy/3920-1/2005 indicate that it represents a new avian paramyxovirus (APMV-12). *Archives of Virology*, 158, 2233–2243.

Usachev, E., Fediakina, I., Shchelkanov, M., Vov, D., Prilipov, A.,& Iamnikova, S. (2006). Molecular genetic characteristics of the Newcastle Sterna/Astrakhan/Z275/2001 virus isolated in Russia. *Molecular Genetics, Microbiology and Virology*, 1, 14–20.

Wakamatsu, N., King, D.J., Kapczynski, D.R., Seal, B.S., & Brown, C.C. (2006). Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002-2003. *Veterinary Pathology*, 43, 925–933.

Zhang, P., Xie, G., Liu, X., Ai, L., Chen, Y., Meng, X., … Yin. R. (2015). High Genetic Diversity of Newcastle Disease Virus in Wild and Domestic Birds in Northeastern China from 2013 to 2015 Reveals Potential Epidemic Trends. *Applied and Environmental Microbiology*, 82, 1530–1536.

Zhu, J., Hu, S., Xu, H., Liu, J., Zhao, Z., Wang, X. & Liu. X. (2016). Characterization of virulent Newcastle disease viruses from vaccinated chicken flocks in Eastern China. *BMC Veterinary Research*, 12, 113.

Table 1. Epidemiological details of the Genotype XIII NDVs isolated from Southern India

Name of the isolate	Type of bird	Age of the bird (Weeks)	Flock size	Month/ Year of isolation	Mortality (%)	Region of isolation	HI titer (Log2)	MDT (hrs)	Fusion protein cleavage sequence	GenBank accession no.
PDDSL-1	Layer chicken	36	8000	04/2015	13.0	Namakkal, TN, India	1024	48	RRQRR/F	MF362982
PDDSL-2	Layer chicken	28	8000	04/2015	8.0	Namakkal, TN, India	256	48	RRQRR/F	MF362983
PDDSL-3	Colour broiler	11	7500	05/2015	77.0	Palladam, TN, India	1024	48	RRRKR/F	MF362984
PDDSL-4	Colour broiler	11	7500	05/2015	62.0	Palladam, TN, India	512	48	RRRKR/F	MF362985
PDDSL-5	Colour broiler	11	7500	05/2015	43.0	Palladam, TN, India	1024	54	RRRKR/F	MF362986
PDDSL-6	Backyard chicken	28	100	08/2015	80.0	Karur, TN, India	512	48	RRQKR/F	MF362987
PDDSL-7	Backyard chicken	3	10	06/2016	60.0	Trichangode, TN, India	128	54	RRRKR/F	MF362988

Table 2. Primers used for amplification and sequencing of the fusion protein gene of the seven isolates

Forward primer sequence	**Reverse primer sequence**	**Amplicon size**
CACTAAGATAGAGAAGAG GCACACC	TTATACAGTCCAATTCTCGCGCC	815 base pairs (bp)
AAAGAGGCATGTGCAAAAGCCCC	GGTGTAGTGAGTGCACCTTCAGTCT	929 bp
GGGAGCCTAAATAATATGCGCGCC	GCG CCA TGT ATT CTT TGC TTC	1058 bp

Table 3. Comparison of amino acid substitutions within the functional domains of fusion protein sequence

Strains	**Fusion peptide (117-141)**		**HRa (143-185)**		**HRb (268-299)**	**HRc (471-500)**		**Transmembrane domain (501-521)**				
	118	**139**	**153**	**170**	**270**	**479**	**494**	**509**	**511**	**513**	**514**	**517**
Consensus of all genotypes	I	A	R	D	T	D	K	V	S	V	F	L
NDV strain LaSota	-	-	-	-	-	N	-	I	-	-	-	-
PDDSL-1	-	-	-	N	A	-	R	A	S	A	-	-
PDDSL-2	-	-	-	N	A	-	R	A	S	A	-	-
PDDSL-3	-	-	-	-	A	-	R	A	S	I	S	-
PDDSL-4	-	-	-	-	A	E	R	A	-	I	S	-
PDDSL-5	-	-	-	-	A	-	R	A	-	I	S	-
PDDSL-6	-	-	-	-	A	-	R	A	-	A	-	-
PDDSL-7	-	-	-	-	A	-	R	A	-	-	S	-

-, no change in amino acid compared to that of CONSENSUS

Table 4. Comparison of F protein amino acid changes at the hypervariable region, fusion protein cleavage site, neutralizing epitopes and glycosylation sites

Strains	Hypervariable region																	Fusion protein cleavage site						Neutralizing epitopes		
	4	10	11	13	14	16	17	20	22	25	26	27	28	29	30	31	69	112	113	114	115	116	117	72,74,75,78,79	157-171	343
B1	R	P	A	M	M	T	I	A	V	C	I	C	P	A	N	S	L	G	R	Q	G	R	L	DEAKA	SIAATNEAVHEVTDG	L
LaSota	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-----	----------	-
Mukteswar	-	-	V	L	-	-	-	T	A	Y	V	R	L	T	S	-	M	R	-	-	R	-	F	-----	------G--------	-
PDDSL-1	K	-	V	L	R	I	-	M	I	-	-	-	L	T	S	-	I	R	-	-	R	-	F	--E--	----------N-	-
PDDSL-2	K	-	V	L	R	I	-	M	I	-	-	-	L	T	S	-	I	R	-	-	R	-	F	--E--	----------N-	-
PDDSL-3	K	-	I	L	G	I	T	M	I	-	-	-	L	T	S	-	-	R	-	R	K	-	F	-----	----------	-
PDDSL-4	K	-	I	L	G	I	T	M	I	-	-	-	L	T	S	-	-	R	-	R	K	-	F	-----	----------	-
PDDSL-5	K	-	I	L	G	I	T	M	I	-	-	-	L	T	S	-	-	R	-	R	K	-	F	-----	----------	-
PDDSL-6	K	-	V	L	W	I	T	M	I	-	-	-	L	T	S	-	I	R	-	-	K	-	F	-----	----------	-
PDDSL-7	K	L	I	L	G	I	T	M	I	-	-	-	L	T	S	-	I	R	-	R	K	-	F	-----	----------	-

-, no change in amino acid compared to that of NDV strains B1 and LaSota
Amino acid matching with that of Mukteswar strain are highlighted in grey

Table 5. Estimates of Evolutionary Divergence between sequences of strains of genotype XIIIe isolates

	PDDSL-3	**PDDSL-4**	**PDDSL-5**	**PDDSL-7**
PDDSL-3		(0.002)	(0.001)	(0.004)
PDDSL-4	0.006		(0.001)	(0.004)
PDDSL-5	0.002	0.004		(0.004)
PDDSL-7	0.029	0.029	0.027	

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by bootstrap procedure (500 replicates). Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 4 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Table 6. Estimates of evolutionary divergence between subgenotypes of genotype XIII viruses

Subgenotypes of Genotype XIII	XIIIa	XIIIb	XIIIc	XIIId	XIIIe	XIII subcluster I	XIII subcluster II
XIIIa		(0.016)	(0.021)	(0.013)	(0.023)	(0.022)	(0.018)
XIIIb	0.104		(0.022)	(0.010)	(0.013)	(0.025)	(0.019)
XIIIc	0.137	0.153		(0.021)	(0.026)	(0.029)	(0.028)
XIIId	0.074	0.056	0.132		(0.016)	(0.024)	(0.019)
XIIIe	0.135	0.076	0.159	0.082		(0.033)	(0.026)
XIII subcluster I	0.129	0.155	0.171	0.129	0.189		(0.026)
XIII subcluster II	0.120	0.130	0.194	0.116	0.162	0.157	

The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal and obtained by bootstrap procedure (500 replicates). Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 58 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 354 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Evolutionary distance between XIIIe and other subgenotypes of genotype XIII are compared.