Haryana Vet. (June, 2022) **61(1),** 46-50

Research Article

MOLECULAR CHARACTERIZATION OF CIRCULATING NEWCASTLE DISEASE VIRUS FROM POULTRY

NISHANTH, C.[1], PRADEEP KUMAR KAPOOR[1], NAVEEN KUMAR[2], RIYESH THACHAMVALLY[2], SANJAY BARUA[2] and NARESH JINDAL[1]*

[1]Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125004

[2]National Centre for Veterinary Type Cultures, ICAR-National Research Centre on Equines, Hisar-125001

Received: 04.06.2021; Accepted: 19.03.2022

ABSTRACT

The present study was conducted to isolate and characterize Newcastle disease virus (NDV) from commercial broiler chicken and layer birds and backyard poultry. Samples were collected from 85 poultry flocks suspected for Newcastle disease (ND). Virus isolation was done in 9-day-old specific pathogen free (SPF) embryonated chicken eggs and the virus isolation was confirmed by haemagglutination (HA) assay. All the HA positive allantoic fluid (isolates; n=23) were found positive for NDV-specific genome by PCR. PCR-RFLP using restriction enzymes *Bgl*I and *Hha*I- analysis of F gene revealed that 22 isolates belonged to lentogenic type (all from commercial poultry) and one was velogenic (from backyard poultry). On deduced amino acid sequence analysis of Fusion protein cleavage site, 21 of 22 lentogenic isolates revealed 112-GRQGRL-117 motif; while one lentogenic isolate revealed novel sequence at cleavage site 112-RRQKRS-117 and the velogenic isolate revealed 112-RRQKRF-117 sequence motif. On phylogenetic analysis, velogenic isolate clustered with Indian genotype XIII (class II velogenic NDVs) with 97.8% similarity at the amino acid level. Of the 22 isolates from commercial poultry, 21 clustered with genotype II of class II lentogenic NDVs with an amino acid sequence similarity of 88-100%; the remaining one isolate was lentogenic (by RE analysis), however, it clustered with R2B strain (mesogenic) with 97.8% sequence similarity. Moreover, it had F117S mutation in its Fusion protein. These results indicate that NDV strains of different genotypes are circulating in commercial and backyard poultry in Haryana (India) and further study are needed on F117S substitution.

Keywords: Newcastle disease virus, Pathotyping, Poultry, Restriction enzyme analysis, RT-PCR

How to cite: Nishanth, C., Kapoor, P.K., Kumar, N., Thachamvally, R., Barua, S. and Jindal, N. (2022). Molecular characterization of circulating newcastle disease virus from poultry. *Haryana Vet.* **61(1)**: 46-50.

Newcastle disease (ND) is one of the economically important viral diseases of the domestic and wild birds around the world. Birds of all age and breeds are susceptible to infection with Newcastle disease virus (NDV). The disease has drastically affected the economy of poultry industry. Newcastle disease is caused by an avian paramyxovirus (APMV), which has a non-segmented, negative sense, single stranded RNA genome. The NDV has been divided into two classes i.e., Class I and Class II. NDVs belonging to Class I are present mostly in wild birds and are generally avirulent in nature. Class II viruses are more prevalent among domestic poultry and are more virulent as compared to class I NDVs. Based on complete fusion (F) gene sequences, Class I and Class II NDVs were grouped into genotypes and sub-genotypes.

There has been phenomenal growth of poultry in India in last two decades; the state of Haryana has made rapid strides during this period in all three segments i.e., breeders, commercial layers and commercial broiler chicken. For the proper implication of the control program against ND, it is important to understand the genetic diversity of NDV strains circulating in the local vicinity. We report the isolation and molecular characterization of NDVs from commercial poultry in Haryana, India.

*Corresponding author: nareshjindal1@gmail.com

MATERIALS AND METHODS

Virus Isolation: A total of 85 samples (pool of lungs, trachea, and spleen) suspected for ND were collected from both commercial and backyard poultry presented at the Disease Investigation Laboratory of the University. Of the 85 specimens, 65 belonged to commercial broiler chicken flocks (45 flocks were vaccinated), 15 from layers (vaccination status of 12 were known), and five from backyard poultry (all unvaccinated). One flock represented one sample; thus, the samples were collected from 85 flocks suspected for ND. The clinical and/or post-mortem findings included torticollis, greenish diarrhoea, petechial haemorrhages at the tip of proventricular papillae, caecal haemorrhages and/or respiratory problems.

A 10% suspension of the sample was made in phosphate buffer saline (PBS) containing antibiotic-antimycotic solution. The clarified supernatant was filtered through 0.45µ filter and used for virological assays. For virus isolation, the sample was inoculated via allantoic route in 9-days old specific pathogen free (SPF) embryonated chicken eggs. Death within 24 hours was indicative of bacterial contamination. Embryos died following virus inoculation were examined for the presence of characteristic lesions of ND. Allantoic fluid was harvested at 3 days post inoculation and virus isolation

was confirmed by haemagglutination (HA) assay. Two additional passages in SPF eggs were performed to confirm whether the virus is replication competent or not.

Reverse Transcription-Polymerase Chain Reaction: HA-positive allantoic fluid were processed for RNA extraction using TRI reagent (Sigma, India) as per the manufacturer's instructions followed by cDNA synthesis (reverse transcription) and amplification of NDV-specific gene (F) segment in PCR by using the primers: forward primer 5'-GCAGCTGCAGGGATTGTGGT-3' and reverse primer 5'- TCTTTGAGCAGGAGATGTTG-3' flanking fusion gene of the NDV (Nanthakumar *et al.*, 2000). For PCR amplification, each 25 µl reaction mixture contained 12.5 µl master mix, 1 µl each forward and reverse primer (20 µM), 2.5 µl dye (coral load), 6.5 µl nuclease free water and 1.5 µl template. The reaction conditions were: initial denaturation for 5 min at 95 °C; 35 cycles of 50 sec at 95 °C (denaturation), 50 sec at 55 °C (annealing) and 50 sec at 72 °C (extension); and a final extension step of 10 min at 72 °C. The amplified PCR products were run in 1% agarose gel. Commercially available vaccines (F, LaSota and R2B strains) were used as positive controls.

Restriction Endonuclease (RE) Analysis: F gene-specific PCR product (356 bp) was purified using QIAamp Viral RNA Mini kit (Qiagen, USA). Approximately 1 µg of the gel purified PCR product along with positive controls (F, LaSota and R2B vaccines) were subjected to digestion with *Bgl*I and *Hha*I (New England Biolabs) at a constant temperature of 37°C for 4 hrs. Digested products were run on 1% agarose gel.

Nucleotide Sequencing: Purified PCR products were subjected to nucleotide sequencing at Sanger's platform using forward and reverse PCR primers. High quality sequences were analyzed at NCBI (http://www.ncbi.nlm.nih.gov) using BLAST to confirm their identity. The nucleotide sequences were aligned using ClustalW method of MEGA 6 software. The evolutionary distances were computed by pair-wise distance method using the Maximum Composite Likelihood Model. The phylogenetic tree of aligned sequences was constructed by Neighbor-Joining method; 295 nucleotide long (nt 170 to nt 465) F gene sequences were selected for alignment after contig formation and trimming. The nucleotide sequences were translated to deduce amino acid sequences (amino acid residue in F protein from position 66 to 150). Percent identity of different sequences was determined using DNASTAR Lasergene software. The sequences were then submitted to GenBank database.

RESULTS AND DISCUSSION

Isolation and PCR: Of the 85 flocks, only 23 were found HA positive (allantoic fluid). All the HA positive allantoic fluid also showed desired NDV-specific amplification in PCR (Fig. 1). There may be involvement of other pathogens in the remaining 62 flocks, which needs further investigation. It is also possible that few of the specimens compromised with their quality i.e., virus titer, and/or survivability, both of which can affect virus isolation. Direct testing of such specimens by RT-PCR (which was not attempted in this study) might have detected many of them if not all as NDV positive.

RE Analysis: In this study, PCR product from F and LaSota vaccine strains and 22 out of 23 field isolates produced two fragments of size 116 bp and 240 bp upon *Bgl*I digestion suggesting these 22 field isolates were of lentogenic type (Fig. 2). Like R2B vaccine strain, one of the isolates (HR-23) remained intact upon *Bgl*I digestion, suggesting it may be mesogenic/velogenic pathotype (Fig. 3). *Hha*I digestion of mesogenic strains is expected to produce two fragments of 116 bp and 240 bp; *Hha*I digestion cleaved the R2B vaccine strain but not field strain (HR-23) suggesting this field isolate may belong to velogenic pathotype (Figs. 3, 4).

PCR-RE is routinely used to differentiate NDV pathotypes (Ballagi-Pordany *et al.*, 1996; Nanthakumar *et al.*, 2000). *Bgl*I differentiates the lentogenic strains (F and LaSota) from the mesogenic (R2B) and velogenic strains, while the restriction enzyme *Hha*I differentiates mesogenic and lentogenic strains from velogenic strains. Twenty-two isolates from commercial broilers and layer birds appeared to be lentogenic in nature while the isolate from backyard poultry was velogenic in nature. Many workers have used different restriction enzymes such as *Alu* I, *Apa* I, *BstO* I, *Hae* III, *Hinf* I, *Pst* I, *Nar* I and *Rsa* I to differentiate virulent and avirulent NDV isolates (Ballagi-Pordany *et al.*, 1996; Mase and Kanehira, 2012). This previously established technique was proven satisfactory in classifying NDV isolates into velogenic, mesogenic and lentogenic strains.

Sequence Analysis and Phylogenetics analysis: Fusion protein cleavage site (FPCS; amino acid residues 112-117) from 21 of the 23 isolates (HR-01 to HR-21) had a lentogenic type sequence motif *viz*; 112-GRQGRL-117. One of the isolates had sequence motif of 112-RRQKRS-117 (HR-22) (mesogenic type) whereas the remaining one (HR-23) had 112-RRQKRF-117 (velogenic type) (Table 1, Fig. 5). The nucleotide sequence of NDVs of this study revealed that the field isolate HR-23 had two unique nucleotide substitutions (G289C and G347A) while the isolate HR-22 had one nucleotide change (T366 C) with respect to the other NDVs of this study (Fig. 5). The isolate HR-23 had one unique amino acid change i.e., arginine at

Table 1

Summary of the NDV results by different methods

Sr.No.	Isolate ID	HA results	PCR results	Restriction digestion		Amino acid sequence	Pathotype
				Bgl1	Hha1		
1.	NDV/Poultry/Egg/India/2015/HR-1 to NDV/Poultry/Egg/India/2015/HR-20	+	+	+	+	112-GRQGRL-117	Lentogenic
2.	NDV/Poultry/Egg/India/2016/HR-21	+	+	+	+	112-GRQGRL-117	Lentogenic
3.	NDV/Poultry/Egg/India/2015/HR-22	+	+	+	+	112-RRQKRS-117	Mesogenic?
4.	NDV/BYP/Egg/India/2016/HR-23	+	+	-	-	112-RRQKRF-117	Velogenic

position 111 preceding to Fusion protein cleavage site (FPCS) whereas one unique substitution (F117L, mesogenic/ velogenic) was observed in isolate HR-22. The sequences were submitted to GenBank database under accession numbers KX688760- KX688780.

Based on FPCS, 21 of the 23 isolates of this study had a sequence motif of lentogenic type. One of the isolates (HR-22 with sequence motif of 112-RRQKRS-117 suggested this isolate to be mesogenic/velogenic in nature while sequence motif of 112-RRQKRF-117 in one isolate (HR-23) suggested it to be velogenic in nature. Presence of lentogenic strains in commercial poultry could be attributed either to the use of live vaccines or field lentogenic strains circulating in the environment.

The isolate (HR-22) showing substitution of serine at position 117 belonged to an unvaccinated commercial broiler flock, though impact of this substitution needs further studies. This substitution appears to be a unique as the presence of phenylalanine in virulent strains and leucine in lentogenic strains has been reported (Alexander and Senne, 2008). Based on FPCS from 112-116, this isolate appeared to be velogenic/mesogenic, however, RE digestion pattern suggested this isolate to be lentogenic as it had restriction site for *Bgl*I. Failure of the restriction enzyme *Bgl*I to differentiate between avirulent and virulent strains has been reported previously (Cattoli *et al.*, 2011; Mase and Kanehira, 2012). Further studies are required to characterize this isolate and its ability to induce the disease in poultry. HR-23 isolated from backyard poultry had amino acid arginine at position 111 prior to FPCS; a similar substitution has been reported in an NDV (Pandu strain) isolated in North-Eastern part of India. The NDV isolate (Pandu strain) was reported to be a novel velogenic pathotype of NDV (Nath *et al.*, 2016).

On phylogenetic analysis (partial F gene based) all the isolates under this study belonged to class II NDVs. Isolate HR-23 clustered with NDV/Chicken/India/2015/ Pandu (KT 734766) strain from India (Fig. 6); this strain belongs to class II, genotype XIII showing an identity of

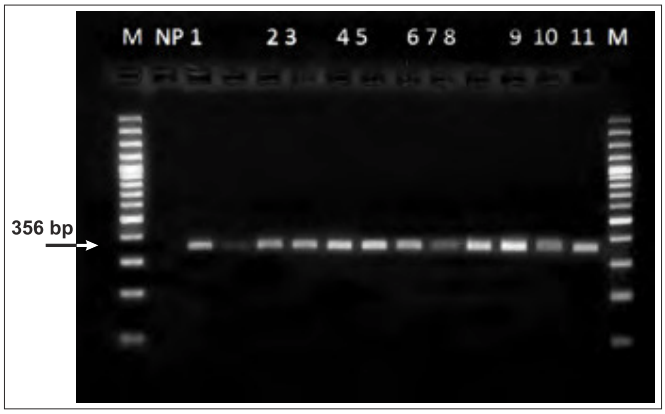

Fig. 1. Amplification of partial 'F' gene of Newcastle disease virus by RT-PCR. Lane M=100bp DNA ladder as molecular size marker; Lane N=Negative control; Lane P=Positive control; Lanes 1 to 11=Field isolates of NDV

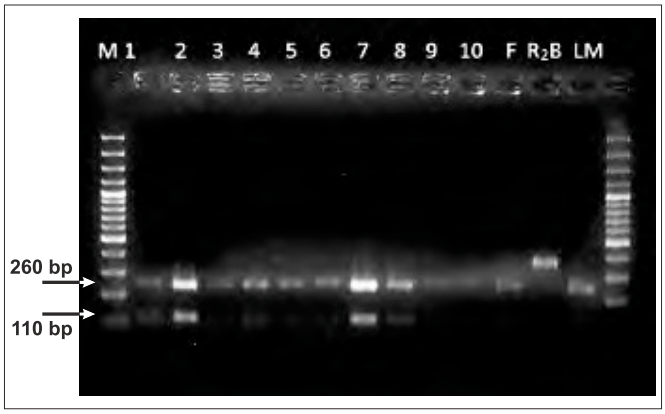

Fig. 2. PCR-RE analysis (*Bgl*I) to differentiate avirulent from virulent strains of Newcastle disease virus Lane M=100bp DNA ladder as molecular size marker; Lanes 1- 10=Field isolates; Lane F=F vaccine strain; Lane R2B=R2B vaccine strain; Lane L=LaSota vaccine strain

95.7% at the nucleotide level. The field isolates HR-01 to HR-21 clustered with lentogenic strains (class II, genotype II) with a nucleotide sequence similarity of 97% to 100%. HR-22 showed the highest similarity of 96% with the vaccine strain R2B (class II, genotype III). The amino acid sequence alignment showed more or less similar pattern to that of nucleotide sequence alignment. Evolutionary distance analysis indicated that the NDVs of this study could be classiûed into genotypes II and XIII. The HR-23

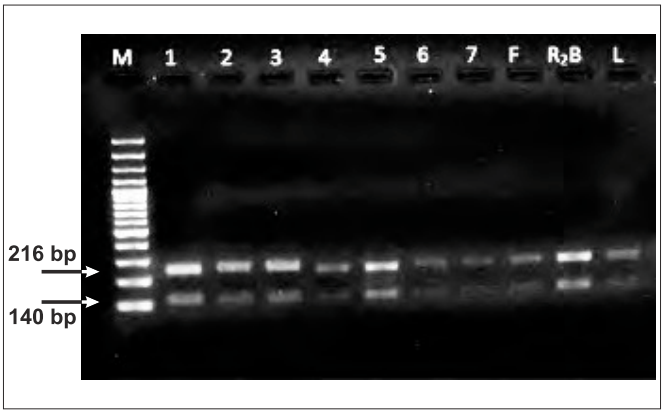

Fig. 3. PCR-RE analysis (*Hha*I) to differentiate lentogenic and mesogenic from velogenic strains. Lane M=100bp DNA ladder as molecular size marker; Lanes 1-7=Field isolates; Lane F=F vaccine strain; Lane R2B=R2B vaccine strain; Lane L=LaSota vaccine strain

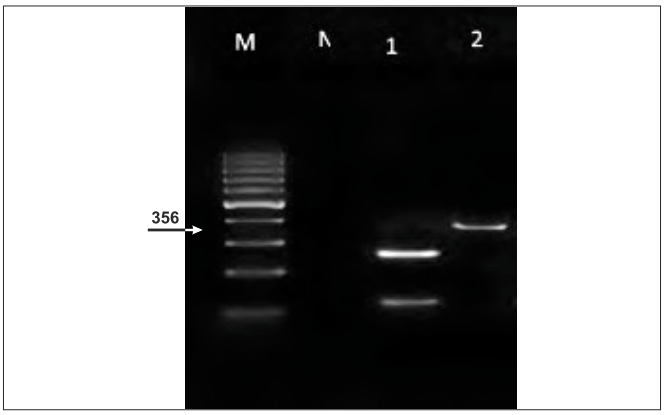

Fig. 4. PCR-RE analysis of R2B vaccine strain and HR-23 isolate with *Hha* I enzyme. Lane M=100bp DNA ladder as molecular size marker; Lane N=Non template control; Lane 1=R2B vaccine strain digested with *Hha* I; Lane 2= HR-23 isolate digested with *Hha* I.

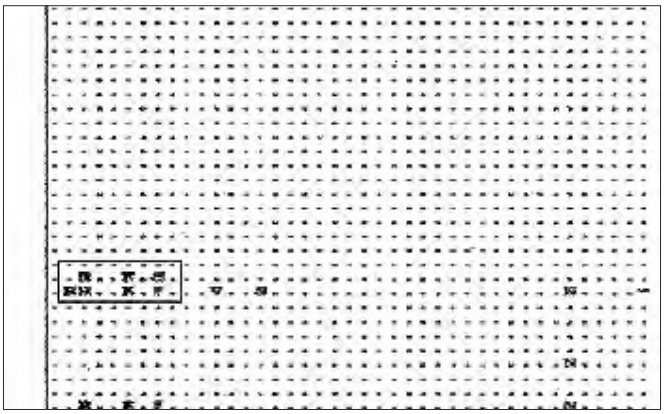

Fig. 5. Alignment of deduced amino acid sequences flanking cleavage activation site of fusion gene of NDV. The boxed sequence at the bottom indicates the amino acid positions (112-117).

NDV isolate of this study clustered with Pandu strain of NDV; a genotype XIII NDV. Genotype XIII viruses were previously classiûed as genotype VII in India and comprise virulent viruses that have been isolated from different countries including India (Xue *et al.*, 2017; Gowthaman *et al.*, 2019; Mariappan *et al.*, 2018). The genotype XIII

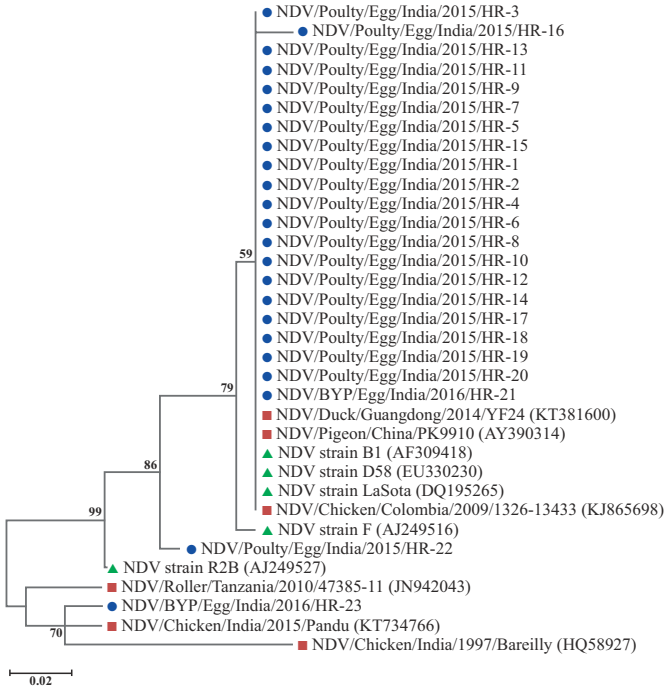

Fig. 6. Phylogenetic tree based on amino acid sequence of fusion gene of Newcastle disease virus. The evolutionary history was inferred using the Neighbor-joining method. The optimal tree with the sum of branch length=0.275000 is shown. The percentage of replicate trees in which the associated taxa clustered together in bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using p-distance method and are in the units of number of amino acid substitution per site. The analysis involved 34 amino acid sequences. All position containing gaps and missing data were eliminated. Blue colour circle indicates field isolates of this study, green triangle indicates known vaccine strains, Orange square indicates already reported NDV strains used for comparison in this study.

isolate of our study was from backyard poultry. Though we could not test the pathogenicity of these isolates in vivo, previous study (Lei-Tao *et al.*, 2008) suggests that some of the virulent NDV isolates may have lentogenic type sequence motif.

Newcastle disease is a major hindrance for the successful proûtable poultry production. Increased reports of ND and/or circulation of NDVs in backyard poultry may pose a threat to the commercial poultry (Dhaygude *et al.*, 2017; Ogali *et al.*, 2018). Backyard poultry has been reported to suffer from virulent NDV (Dimitrov *et al.*, 2016). Due to contact between backyard and commercial poultry, there are chances of virus transmission from backyard to commercial poultry and vice versa. Invariably, backyard poultry is not vaccinated against NDV. Wild birds have been reported to act as carriers for NDV (Lindh *et al.*, 2012). Considering the wide economic impact of virulent NDV outbreaks, extensive surveillance strategies in India should include commercial, backyard poultry and wild birds.

Currently, LaSota, D58, B1, F (lentogenic) or Mukteswar and R2B (mesogenic) are commonly used vaccine strains in poultry. The unexpected similarity among the study isolates with that of the commonly used vaccine strains in this study probably indicates spillover of live NDV vaccines strains.

During NDV surveillance, the importance of vaccine-derived viruses is usually neglected as the emphasis is given only on the identification of pathogenic or virulent isolates as they cause severe outbreaks. There is a need to study the reverse spillover of live agents from domestic poultry to wildlife as a result of the mass usage of live NDV vaccines. It has been found that all of the pathogenic strains/genotypes of NDV belong to one serotype i.e., APMV-1 and upon vaccination using heterologous genotype/strains (from avirulent and less virulent strains i.e., genotypes I and II) can cross protect the birds from the disease, but fail to prevent viral shedding (Kapczynski and King, 2005). In a recent study, combined inactivated Lasota and genotype VIId vaccine was found effective in reducing virus shedding in SPF chicks challenged with genotype VIId strain of NDV (Mahmoud *et al.*, 2019). The NDV has an opportunity to acquire speciûc mutations, which may facilitate evasion of the immune response induced by vaccination (Perozo *et al.*, 2012); several such mutations can arise over time and give rise to new genotypes and strains.

It can be concluded from the present study that multiple NDV genotypes, i.e., genotypes II and XIII are circulating in poultry in the state of Haryana, India. The velogenic strain from backyard poultry shared maximum similarity with NDV isolated from North-eastern part of India. For proper control of NDV, poultry farmers should take strict biosecurity measures to minimize the chances of virus spread from backyard poultry to commercial poultry.

ACKNOWLEDGMENT

The authors are thankful to National Research Centre on Equines, National Centre for Veterinary Type Culture Collection, Hisar, Haryana (India) for their support during the entire research.

REFERENCES

Ballagi-Pordany, A., Wehmann, E., Herczeg, J., Belak, S. and Lomniczi, B. (1996). Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. *Arch. Virol.* **141**: 243-261.

Cattoli, G., Susta, L., Terregino, C. and Brown, C. (2011). Newcastle disease: A review of field recognition and current methods of laboratory detection. *J. Vet. Diagn. Invest.* **23(4)**: 637-656.

Dhaygude, V.S., Sawale, G.K., Chawak, M.M., Bulbule, N.R., Moregaonkar, S.D. and Gavhane, D.S. (2017). Molecular characterization of velogenic viscerotropic Ranikhet (Newcastle) disease virus from different outbreaks in desi chickens. *Vet. World.* **10(3)**: 319-323.

Dimitrov, K.M., Bolotin, V., Muzyka, D., Goraichuk, I.V., Solodiankin, O., Gerilovych, A, Stegniy, B., Goujgoulova, G.V., Silko, N.Y., Pantin-Jackwood, M.J., Miller, P.J. and Afonso, C.L. (2016). Repeated isolation of virulent Newcastle disease virus of sub-genotype VIId from backyard chickens in Bulgaria and Ukraine between 2002 and 2013. *Arch Virol.* **161**: 3345-3353.

Gowthaman, V., Ganesan, V., Gopala Krishna Murthy, T.R., Nair, S., Yegavinti, N., Saraswathy, P.V., Kumar G.S., Udhayavel, S., Senthilvel, K. and Subbiah, M. (2019). Molecular phylogenetics of Newcastle disease viruses isolated from vaccinated flocks during outbreaks in Southern India reveals circulation of a novel sub-genotype. *Transbound. Emerg. Dis.* **66(1)**: 363-372.

Kapczynski, D.R. and King, D.J. (2005). Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* **23**: 3424-3433.

Lei-Tao, T., Huai-Ying, Xu., You-Ling, W., Zhuo-Ming, Q., Lei, S., Wen-Jun, L. and Zhi-Zhong, C. (2008). Molecular characterization of three new virulent Newcastle disease virus variants isolated in China. *J. Clin. Microbiol.* **46**: 750-753.

Lindh, E., Ek-Kommonen, C., Vaananen, V.M., Alasaari, J., Vaheri, A., Vapalahti, O. and Huovilainen, A. (2012). Molecular epidemiology of outbreak-associated and wild-waterfowl- derived Newcastle disease virus strains in Finland, including a novel class I genotype. *J. Clin. Microbiol.* **50**: 3664-3673.

Mahmoud, N.K., El-Deeb, A.H., Emara, M.M., Abd El-Khaleck, M.A. and Hussein, H.A. (2019). Genotypes II and VII d-based inactivated Newcastle disease vaccine reduces virus shedding. *Virus Dis.* **30(3)**: 453-461.

Mariappan, A.K., Munusamy, P., Kumar, D., Latheef, S.K., Singh, S.D., Singh, R. and Dhama, K. (2018). Pathological and molecular investigation of velogenic viscerotropic Newcastle disease outbreak in a vaccinated chicken flocks. *Virus Dis.* **29(2)**: 180-191.

Mase, M. and Kanehira, K. (2012). Simple differentiation of avirulent and virulent strains of avian Paramyxovirus serotype-1 (Newcastle disease virus) by PCR and restriction endonuclease analysis in Japan. *J. Vet. Med. Sci.* **74(12)**: 1661-1664.

Nath, B., Barman, N.N. and Kumar, S. (2016). Molecular characterization of Newcastle disease virus strains isolated from different outbreaks in northeast India during 2014-15. *Microb. Pathog.* **91**: 85-91.

Ogali, I.N., Wamuyu, L.W., Lichoti, J.K., Mungube, E.O., Agwanda, B. and Ommeh, S.C. (2018). Molecular characterization of Newcastle disease virus from backyard poultry farms and live bird markets in Kenya. *International J. Microbiol.* Article ID **2368597**. https://doi.org/10.1155/2018/2368597.

Perozo, F., Marcano, R. and Afonso, C.L. (2012). Biological and phylogenetic characterization of a genotype VII Newcastle disease virus from Venezuela: Efûcacy of ûeld vaccination. *J. Clin. Microbiol.* **50**: 1204-1208.

Xue, C., Cong, Y., Yin, R., Sun, Y., Ding, C., Yu, S., Liu, X., Hu, S., Qian, J., Yuan, Q., Yang, M., Wang, C. and Ding, Z. (2017). Genetic diversity of the genotype VII Newcastle disease virus: identification of a novel VII sub-genotype. *Virus Genes.* **53(1)**: 63-70.